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THE MODULATION OF APOPTOSIS IN TESTICULAR GERM CELLS FOLLOWING TOXICANT-INDUCED CELLULAR STRESS

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THE MODULATION OF APOPTOSIS IN TESTICULAR GERM CELLS FOLLOWING TOXICANT-INDUCED CELLULAR STRESS

by

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Dissertation

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Dedication

To my parents James and Susan and to my wife Mireya

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THE MODULATION OF APOPTOSIS IN TESTICULAR GERM CELLS FOLLOWING TOXICANT-INDUCED CELLULAR STRESS

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Di-(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental toxicant. The active metabolite of DEHP, mono-(2-ethylhexyl) phthalate (MEHP), is ultimately responsible for disrupting the process of spermatogenesis and promoting germ cell death. In addition, this toxicant has been positively correlated with developmental problems such as cryptorchidism, a derangement of the seminiferous tubules, and a syndrome called testicular dysgenesis, leading to reduced sperm number. The potential impact of MEHP on human fertility justifies a detailed investigation into the mechanisms by which this agent causes germ cell death. MEHP is known to directly target and damage the Sertoli cell, a testicular cell whose main function is to support the development of the principle germ cell types from the earliest stem cell to the most mature spermatozoa. This dissertation examines the downstream effect of Sertoli cell damage on germ cell homeostasis and the proteins that modulate the sensitivity of germ cells to

undergo apoptotic elimination. Specifically, the stabilization of the p53 protein is proposed to be an important upstream determinant of Fas-mediated apoptosis in germ cells following MEHP exposure. Furthermore, that the resulting cell death is the result of increased death receptor expression and c-FLIP$_L$ ubiquitinylation. The mechanism is speculated to reside in the spermatocyte sub-population of germ cells, which appears to be most responsive to changes in apoptosis.

Exposures of wild type mice to MEHP caused an increased p53 stability and elevated protein levels of the membrane-bound death receptors Fas and DR5 in testicular spermatocytes. The expression of these proteins occur coincident with increases in spermatocyte apoptosis and are driven by p53 activity. To further assess the mechanisms responsible for the sensitivity of germ cells to undergo p53-mediated apoptosis, we used the germ cell line GC-2spd(*ts*) (a p53 temperature sensitive spermatocyte-like cell line that allows for p53 nuclear localization at 32°C but not 37°C). Induction of the p53 protein led to higher levels of the death receptors DR5 and Fas, activation of caspase-8, and decreases in c-FLIP$_L$. Addition of TRAIL (the cognate ligand for DR5) and the agonistic DR5 agonistic antibody MD5-1, triggered a robust synergistic increase of apoptosis in GC-2 cells maintained at the p53 permissive temperature (32°C). DR5 levels on the germ cell plasma membrane were considerably enhanced following these treatments. Immunoprecipitation of c-FLIP$_L$ suggests that the protein is ubiquitinylated after cellular stress and concomitant with p53 activity. Experiments also reveal that c-FLIP$_L$ levels may be influenced by Itch, a regulatory protein able to label targets for the proteasomal degradation using a ubiquitinylating E3 ligase. Immunohistochemical detection in adult wild type mouse testis show robust increases in Itch protein levels upon MEHP treatment (1g/kg) and subsequently localization to the cytoplasm of meiotic spermatocyte germ cells. Western blot analysis of testis from MEHP treated mice also show a correlation between the reduction of c-FLIP$_L$ and an increase in Itch threonine-222 phosphorylation, a necessary modification for its E3 ligase function. These results provide a possible model in which the removal of Sertoli cell support

promotes germ cell death through the extrinsic pathway, ultimately leading to disruption of spermatogenesis and testicular dysgenesis in mammals. However, removal of Itch also show increases in apoptosis and Itch protein deficient mice demonstrate defects in meiosis. Thus, Itch may also play a novel role in the cell cycle.

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List of Abbreviations

AIF	Apoptosis Inducing Factor
APAF-1	Apoptotic Protease Activating Factor-1
ATP	Adenosine Triphosphate
BCL-2	B-cell Lymphoma-2 Gene
BH Domain	BCL-2 Homologous Domain
BIR Domain	Bacuolovirus IAP-Repeat Domain
Caspase	Cysteine Aspartyl-specific Protease
CED	Cell Death Abnormal
CAD	Caspase-Activated DNAse
DD	Death Domain
DED	Death Effector Domain
DEHP	Di-2-(ethyl-hexyl) Phthalate
DISC	Death-Inducing Signaling Complex
DR	Death Receptor
FADD	Fas-Associated Death Domain
FITC	Fluorescein Isothiocyanate
FLIP	FADD-like ICE Inhibitory Protein
FSH	Follicle Stimulating Hormone
GC2(*ts*)	Germ Cell Spermatid (temperature sensitive)
Gld	Generalized Lymphoproliferative Disease
IAP	Inhibitor of Apoptosis Protein
ICAD	Inhibitor of Caspase-Activated DNAse
HECT Domain	E6-AP Carboxyl Terminus Domain
JNK	C-Jun N Terminal Kinase
Lpr	Lymphoproliferation
MAP Kinase	Mitogen-activated Protein Kinase
MDM-2	Mitotic Double Minute-2

MEHP	Mono-2-(ethyl-hexyl) Phthalate
MOMP	Mitochondrial Outer Membrane Permeabilization
PI	Propidium Iodide
PLAD	Pre-ligand Assembly Domain
PT Pore	Permeability Transmission Pore
RING Domain	Really Interesting New Gene Domain
RIP	Receptor-Interacting Protein
ROS	Reactive Oxygen Species
SiRNA	Small Interfering RNA
TDS	Testicular Dygenesis Syndrome
TNFα	Tumor Necrosis Factor Alpha
TNFR	Tumor Necrosis Factor Receptor
TRAIL	TNF-related Apoptosis Inducing Ligand
VDAC	Voltage Dependent Anion Channel

Chapter 1:
Introduction

1.1 The Testis and Spermatogenesis

A. Basic Testis Physiology

Spermatogenesis is the process of producing mature, haploid spermatozoa for fertilization of the female egg. The sites of gamete production are the testes; round, capsule-like paired organs located externally in most mammals. Exceptions include whales and some land mammals, such as elephants, which internalize the testis to maintain the optimal temperature for robust fertility (Gaeth, 1999). Attached to the testis is the epididymis, a tube that stores spermatozoa upon external delivery *via* ejaculation.

An individual testis is divided into two regions: the interstitial compartment, which contains blood and interstitial fluid, and the seminiferous tubules, which contain testicular cells organized in a defined architecture (**Fig. 1.1**). The testicular interstitial compartment contains primarily Leydig cells, which reside in the spaces between individual seminiferous tubules. This Leydig cell is the testicular site of steroid synthesis and is the principle supplier of testosterone in the testis (Zirkin, 2000). The interstitial space also contains a high number of macrophages, a phagocyte important in cell immunity. Macrophages may also aid in the regulation of testosterone levels by releasing reactive oxygen species (ROS) to prevent overexpression of the steroid (Hales, 2002).

Spermatogenesis occurs specifically in the seminiferous tubules of the testis. They are composed of lengthy stretches of tissue that emanate from an external duct (the Rete testis) and are filled with germ cells and Sertoli cells. The tubules form long loops that give the testis their distinct ovular shape. Peritubular myoid cells line the outside of the seminiferous tubules and act to secrete components of extracellular matrix, such as actin filaments, and acts to provide structural integrity and physical support (Tung, 1990). Within the seminiferous tubule compartment is an epithelium that contains two cell types: Sertoli cells and

germ cells. Sertoli cells are of one type, whereas germ cells are divided into four major classes, in the order of least to greatest maturity: spermatogonia, spermatocytes, spermatids, and spermatozoa.

Each testis contains hundreds of closely associated tubules. Each complete individual tubule contains germ cells of all possible maturities (called "stages") and associated Sertoli cells. A cross section of a seminiferous tubule reveals a ring of cells organized in a defined manner according to developmental age, or stage (**Fig. 1.1 & 1.2**). There are twelve stages in the rodent spermatogenic process and each stage marks a place in the development and maturation of germ cells. The least mature cells reside along the outermost basal membrane, while the most mature moves towards the lumen, at the center of the tubule. The proper organization of germ cell types in concert with the Sertoli cell is fundamental to testis physiology and consequently spermatogenesis.

The Sertoli cell is of interest as it acts as the "nurse" to germ cells, providing nutrients, growth factors, and other components vital to their sustained health. The Sertoli cell is able to mitigate this exchange of materials due to its ability to physically contact and interact with up to 30 germ cells at once (Skinner, 2005). Indeed, the cytoplasm of the Sertoli cell is distributed through the layers of adjacent germ cells, stretching from the basement membrane to nearly the lumen. The enveloping character of the Sertoli cell cytoplasm accounts for its wide range of functions. For example, Sertoli cells can phagocytize damaged germ cell material called residual bodies and actively clear other unneeded components from the tubule. However, the Sertoli cell fills numerous other roles, particularly in providing physical support to the germ cell as well as paracrine signaling such as growth factor production.

Sertoli cells produce a tight junction with each other, forming what is sometimes described as the "Sertoli cell barrier" or "blood testis barrier." (Weber, 1988) The tight junctions form two distinct compartments within the seminiferous epithelium, one below the junction (basal) and one above (adluminal) (**Fig. 1.3**). The distinction between the two regions is important because the cells of the basal

compartment have direct access to constituents diffused from the blood and lymphatic fluids. Early germ cell types, including spermatogonia and the least mature spermatocytes, are located in the basal compartment (Russell, 1977). Above the tight junction, in the adluminal compartment, resides the majority of the spermatocytes and all other mature germ cells.

The cells that occupy the region above the tight junction rely, in most cases, completely upon what is secreted by the Sertoli cell for survival. One example is lactate, the principle energy substrate for germ cells, which is delivered exclusively by the Sertoli cell (Jutte, 1982). Additionally, the Sertoli cells control the levels of hormones, such as leutenizing hormone (LH) and follicle stimulating hormone (FSH), both important in controlling differentiation and proliferation of germ cells (Russell, 1990). Sertoli cells regulate FSH balances using activin or its antagonist, inhibin (Meehan, 2000). Increasing FSH leads to augmented levels of androgen-binding protein, testosterone, and 17-β estradiol, inducing spermatogenesis. On the other hand, increased inhibin secretion will prevent this process. Therefore, regulating hormone availability to the germ cell is an example of the paracrine relationship that the two cells feature. Sertoli germs also secrete various factors directly to the germ cells, including anti-Mullerian hormone required for fetal testis development (Boukari, 2007).

Paracrine hormone signaling and nutrient provision is critical to cells located in the adluminal department. However, it is important to emphasize that cells residing within both compartments are reliant on the structural support conferred by the Sertoli cell. The Sertoli cell acts as a scaffold that maintains germ cells in the proper place and orientation for their development throughout spermatogenesis. Elements of the Sertoli cell cytoskeleton are essential for positioning elongate spermatids for spermiation (sperm release) and intermediate filaments called vimentin probably act to orient Sertoli cells with Spermatocytes (Skinner, 2005),(Richburg, 1996). Loss of the proper architecture between the Sertoli cell and germ cell include failure to properly complete spermatogenesis and promotes cell death.

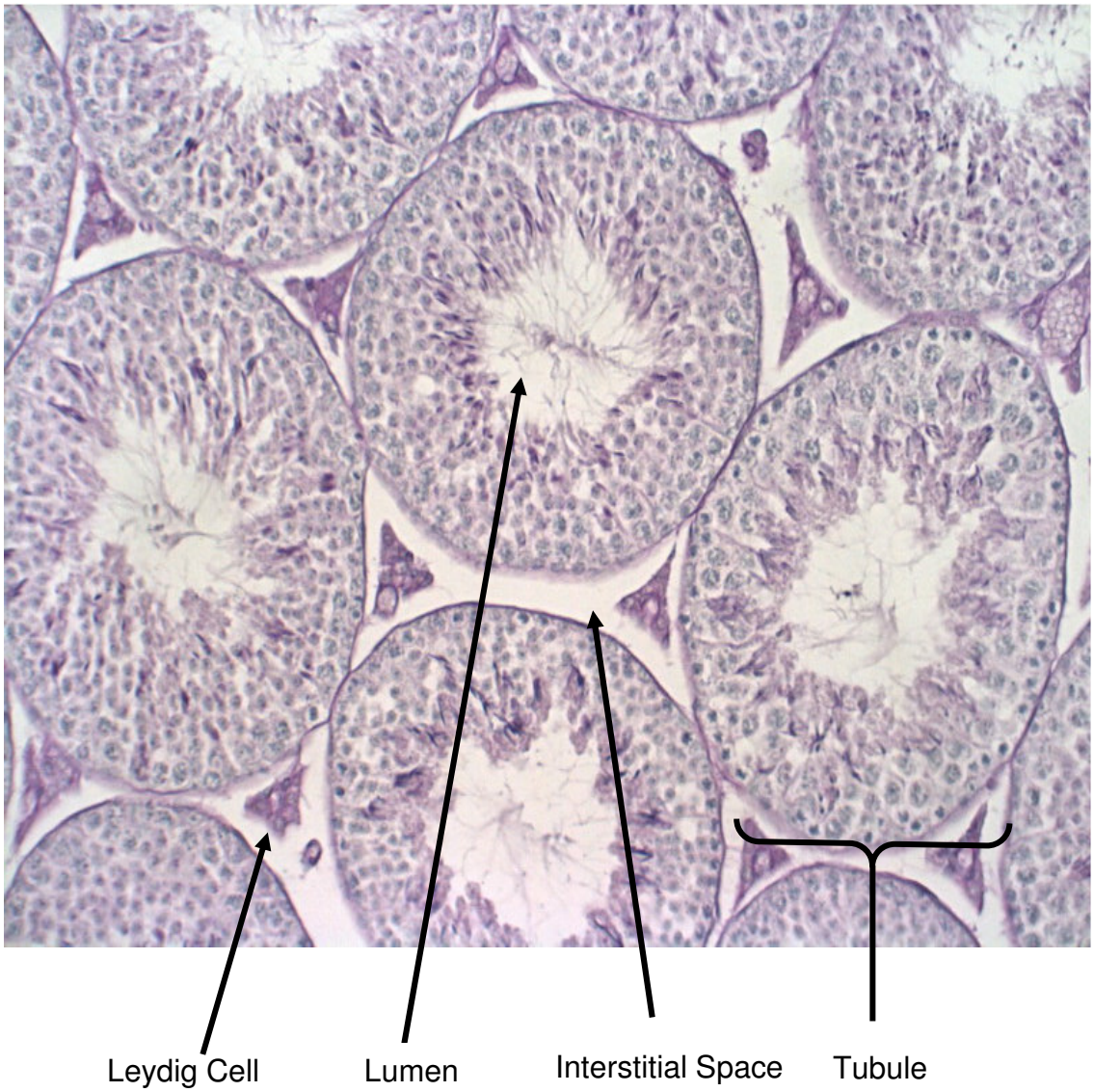

Fig. 1.1 *Cross section of the testis*. The testis is composed primarily of the seminiferous tubules, which contain germ cells and Sertoli cells and is the site of spermatogenesis. The interstitial space exists between the tubules and its main constituents are Leydig cells and macrophages. The lumen is where spermiation occurs and spermatids are released to the epididymis.

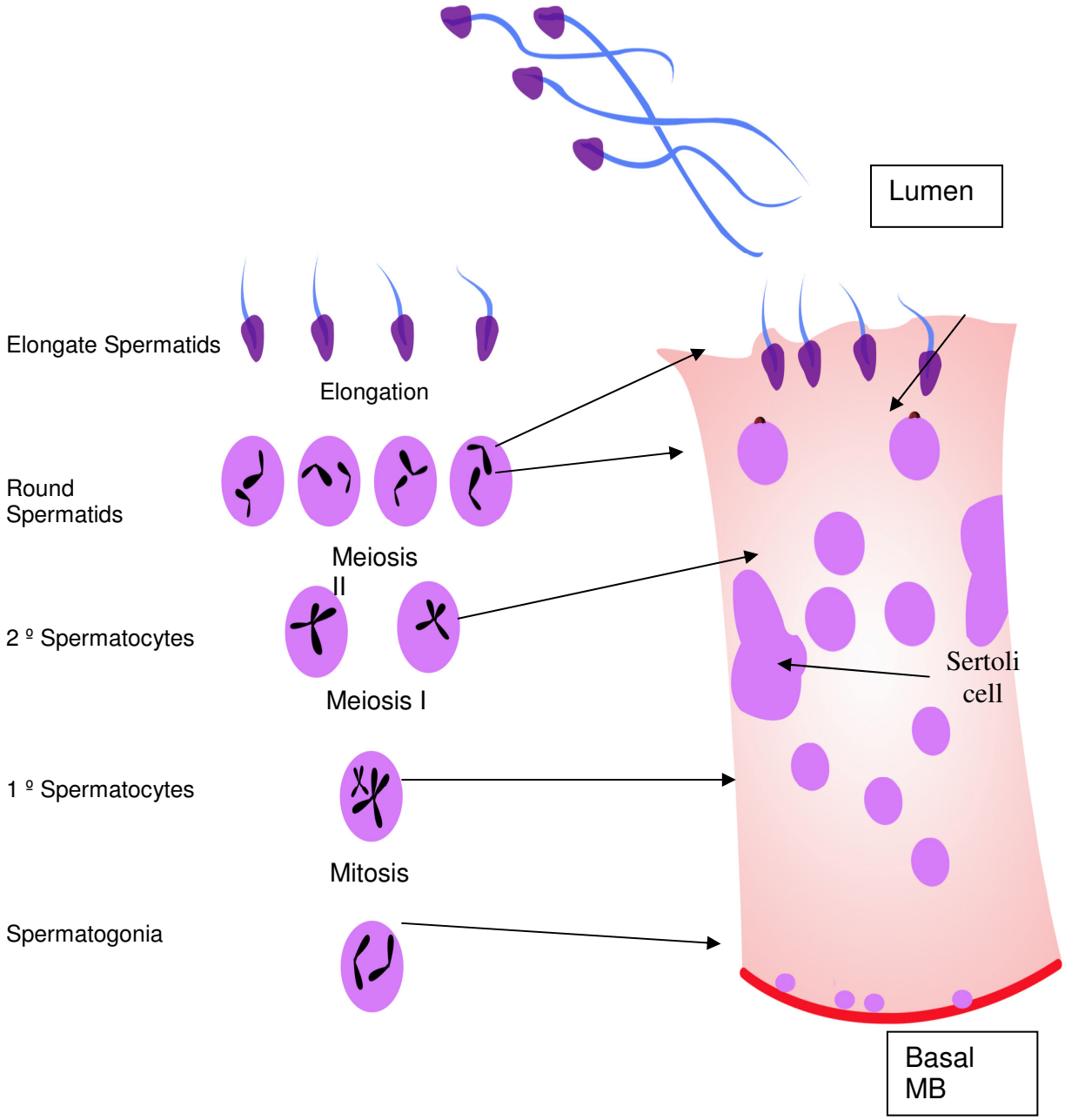

Fig. 1.2 *Germ cell types/stages*. During spermatogenesis, each germ cell either undergoes mitosis or meiosis depending on its maturity. The spermatocyte is the only subtype to undergo meiosis. Round spermatids are recognized by a small acrosome located on end of the cell. Round spermatids elongate and are released from the lumen.

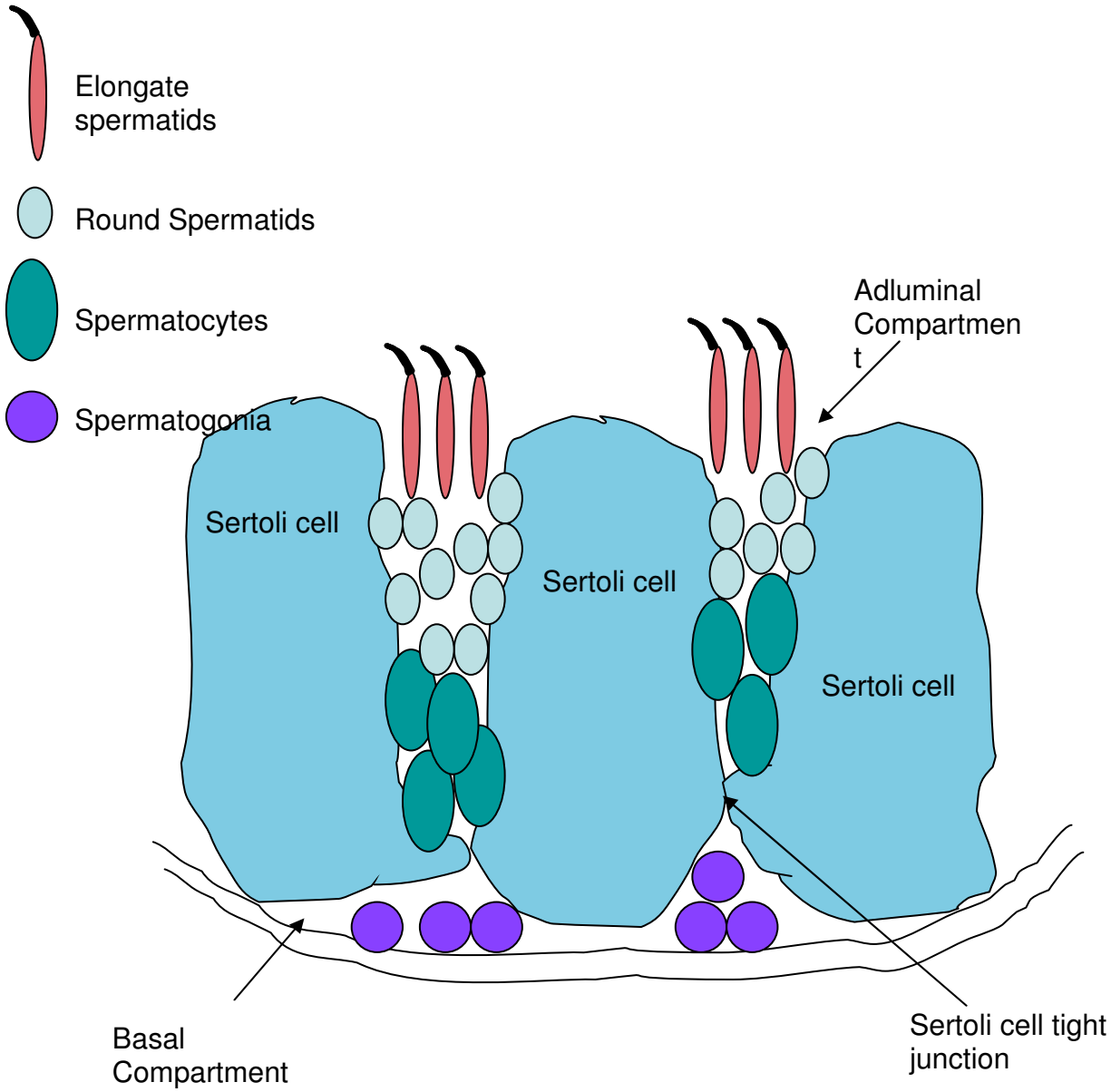

Fig. 1.3 *Germ cell organization***.** Sertoli cells divide the germ cell into two regions: the basal compartment below the tight junctions and the adluminal compartment above the tight junction. Sertoli cell signaling is particular important in this region. Meiosis also occurs in the cells of the adluminal compartment.

B. Germ Cells and Spermatogenesis

Spermatogenesis is marked by the transformation of the germ cell from the early spermatogonia to the fully mature elongate spermatids (spermatozoa are defined after further maturation in the epididymis). The development of mature sperm is a stepwise process that progresses from the basement membrane of the seminiferous tubule to the lumen, where spermiation, or sperm release, occurs. The entire cycle requires precise timing and organization to proceed from the earliest stem-germ cells to the eventual release of spermatozoa. Small defects in timing, cell spacing, or hormone secretion can all result in lower sperm counts, and ultimately result in male infertility. Because cells can become damaged or apoptotic during spermatogenesis, not all germ cells become mature sperm. A pruning of these cells occur under normal physiological conditions, routinely eliminating 75% of the theoretically predicted total germ cell population (Allan, 1972). Even so, healthy mammals typically generate approximately 100 million spermatozoa per day (Aitken, 2006).

The spermatogenic process is initiated with stem spermatagonia that are perpetually maintained at low levels. These most fundamental germ cell types proliferate rarely and give rise to slightly larger type A spermatogonia capable of pairing with each other. Eventually enough paired type A spermatogonia (A_P) are able to align in the tubule, forming long strings of germ cells connected by structures known as intercellular bridges (Weber, 1987). The bridge conformation of consecutive A_P spermatogonia are thought to promote clonality and subsequently divide by mitosis, resulting in the A_1 cell (Huckins, 1978). A series of type A spermatogonia slightly more mature than the previous (designated A_2-A_4 and A intermediate) is produced by cell division before forming the more numerous (and morphologically distinct) type B spermatogonia. All spermatagonia reside along the basement membrane, in the basal compartment, and are considered part of the proliferative phase of spermatogenesis.

The next major germ cell group is the spermatocyte, which represents the meiotic phase of spermatogenesis. It is during this series of events that the genetic content of the gametes are halved, forming haploid cells with 23 chromosomes. Cross-over and genetic recombination also occurs during this developmental stage. The earliest spermatocyte cell types are the preleptotene ("resting") spermatocytes and are the direct product of dividing type B spermatogonia. The preleptotene cell is found in the basal compartment along the basement membrane. However, the later stages of spermatocyte development show movement of the cells in the direction of the lumen, at the center of the tubule. Spermatocytes are able to undergo the first stages of meiosis when they have migrated as far as the adluminal compartment (Skinner, 2005).

The process of meiosis is spread over four temporally controlled periods, in which chromosomes are formed (leptotene), aligned in sister chromatids (zygotene), allowed to undergo recombination *via* cross-over (pachytene), and when the chromosomes separate (diplotein). The pachytene period is the longest, lasting from one to two weeks in most mammals. After meiosis II (in which the genetic material is halved again), the final product is a haploid round spermatid. Leading up to this stage, the germ cells have become progressively larger and rounder in shape. Generally, late stage spermatocytes and round spermatids are among the easiest cells to distinguish morphologically (Russell, 1990).

Round spermatids are gradually extended in shape and project a more pronounced acrosome, which is a specialized compartment emanating from the endoplasmic reticulum filled with lytic enzymes used to breech the egg membrane during fertilization (Austin, 1975). The acrosome is present throughout spermatogenesis but is most easily discernible in spermatids that have undergone an elongation in shape. Elongate spermatids develop into spermatzoa, a mature sperm that possesses a flagellum and motility. At the end of the process, spermatozoa are released from the testis *via* the Rete gland and enter the epididymis for further maturation and storage.

Spermatogenesis is sensitive to small imbalances in endocrine factors and stressors that promote such imbalances. Studies have show that there are actually three quarters less sperm produced than would be theoretically predicted based on division kinetics of the earlier germ cells (Allan, 1992). Much of this difference between the predicted numbers of germ cells and the actual numbers of mature spermatids produced can be attributed to apoptosis of germ cells. A naturally occurring increase in germ cell apoptosis can be seen during specific developmental periods such as during the "first wave" of spermatogenesis that arises during the pre-pubertal period (Rodriguez, 1997). This physiological cell death helps to denote the upper limit of germ cells that Sertoli cells can support and maintains the integrity of spermatogenic processes. Consequently, the number of healthy Sertoli cells will determine the level of germ cell death. Thus, apoptosis is a normal component of spermatogenesis. However, stress induced by toxicants such as MEHP can inappropriately trigger the apoptotic process, leading to reductions in normal levels of gametes and a loss of fertility. The use of MEHP will be discussed as part of the testicular injury model closing this chapter. However, a discussion of the apoptotic process is a necessary precursor to that section.

1.2 Apoptosis

A. General Description and Features

Apoptosis is the process in which cells experience intentional or "programmed" death. The term was first coined by Austrailian researcher John Kerr when he investigated controlled cell deletion as a means of physiological population control (Kerr, 1972). The origin of the term "programmed cell death" actually dates to 1965, when Lockshin and Williams described the apparently designed cell death that occurs during development of tadpole embryos

(Lockshin, 1965). The Greek word apoptosis literally translates to "falling off," or "leaves falling off trees," a metaphor that living matter sometimes dies to accommodate future health.

Apoptosis represents an orderly and efficient dismantling of cells that have been designated for termination. Accidental cell death is referred to as necrosis. Necrosis is a process by which the cell membrane is compromised, leading to a loss of osmotic control and an increased internal pressure within the cell. This pressure will usually culminate in a rupturing of the membrane that disrupts adjacent healthy cells (Krysko, 2006). The lysis of necrotic cells releases DNAses and lytic enzymes that result in inflammation and an immune response that clears necrotic cells and their affected neighbors (Jacotot, 2000). Unlike necrosis, in which injured cells spill their contents in an unorganized manner, apoptosis systematically breaks down the components of a cell and recycles the material.

Kerr *et al* described the basic morphological features of apoptosis: cell shrinkage and loss of contact with adjacent cells, nuclear and cytoplasmic condensation, a budding process by which parts of the cell are wrapped in vesicles known as apoptotic bodies, and eventual phagocytosis *via* the activity of macrophages (Kerr, 1972). Early biochemical markers involve cleavage of CAD (caspase-activated DNase) from its inhibitor, ICAD (Inhibitor of caspase-activated DNase) (Enari, 1998), (Kanouchi, 2005). Released CAD has a nuclear import sequence and is able to identify and cut DNA sequences within the nucleus (Fischer, 2003) (Nagata, 2000). The caspases (cysteine-aspartic acid proteases) responsible for ICAD processing are activated following an apoptotic stimulus and cleave a variety of substrates, including DNA repair proteins (notably PARP – poly ADP-ribose polymersase), cytoskeleton molecules such as actin and vimentin, and the nuclear support protein laminin (Hengartner, 2000). The latter structural protein is of particular importance, as its cleavage promotes the budding of the nuclear contents (Lazebnik, 1993).

One further characteristic of an actively apoptotic cell is the movement of phospholipid component phosphotidylserine from the inside of the plasma membrane to the outside. This lipid "flip-flop" transition occurs prior to the influx of phagocytizing macrophages and does in fact act as a signal to actively recruit those cells (Fadok, 1992). The process is useful for the indication of early apoptosis *versus* necrosis and can be detected in a straightforward cell death assay using flow cytometry.

The first understanding of apoptosis genetics dates to the studies conducted by H. Robert Horvitz's research group a decade after Kerr's description of apoptotic characteristics. Horvitz used the model system *C. elegans*, a hermaphroditic nematode that exhibited an unusual but consistent pattern of death among its somatic cells: exactly 131 of 1090 cells die in the worm during development (Ellis, 1986). Over the course of the next decade the cell death abnormal (*ced*) genes responsible for this phenomena would be identified and their proteins characterized. Inhibitors of cell death would be identified as well. For example, two of the earliest *c. elgans* apoptotic genes to be described was the pro-apoptotic *ced*-3 and the anti-apoptotic *ced*-9. These proteins proved to have similar (though not identicle) human analogs, caspase-9 and Bcl-2 respectively, that could act in an analogous fashion (Yuan, 1993), (Hengartner, 1994). Further studies would illuminate that the family of apoptotic/survival proteins were not restricted to certain organisms and that many of the proteins in fact played a role in the processes of normal development.

Homeostasis and physiological health in any cell is reliant on strict and precise control of apoptotic mechanisms. For example, damaged or otherwise compromised cells are removed by apoptosis for degradation and recovery of usable components like amino acids. Apoptosis can also be selectively employed in the development of structural features in embryos, such as the removal of the membranous webbing between the fingers on hands and feet (Steller, 1995). However, the proper balance of cell survival versus cell death must be maintained by an organism, or catastrophic damage can be incurred.

B. Cell Death Biochemistry

i) Death Receptor Systems and DISC Formation

Apoptosis is mediated through signals internal and external to the cell. The source of the signals vary considerably, but must be able to trigger the series of events that collectively represent the process of cell death. There are two well described apoptotic mechanisms: the intrinsic and extrinsic pathways. While both share certain proteins and biochemical steps, each pathway is initiated and regulated in a distinct manner. Which mechanism is employed is often a function of what signal is introduced into the cell. The intrinsic, or mitochondrial pathway, operates through disruption of the outer mitochondrial membrane and may be engaged by viral infection, reactive oxygen species (ROS), and most chemical toxicants. This pathway can be activated by external signals such as toxicants but the death inducing machinery is contained within the cell.

Conversely, the extrinsic, or death receptor mediated pathway, relies on the interaction between a cell membrane receptor protein and its cognate ligand. The common "death" receptors include Fas (APO-1/CD-95), DR4 (TRAIL-R1), DR5 (TRAIL-R2), and TNFR (tumor necrosis factor receptor) 1 and 2. Their corresponding ligands are FasL, TRAIL (tumor necrosis factor related apoptosis-inducing ligand), and TNF-α (tumor necrosis factor-α) respectively. For the purposes of this dissertation, only the receptor ligand interactions of Fas/FasL and TRAIL/DR5 will be discussed in detail.

Fas and DR5 are trans-membrane proteins expressed on the surface of cells targeted for apoptosis (Itoh, 1991), (MacFarlane, 1997). The proteins are part of a general category of receptors known as the TNF receptor superfamily and contain three conserved domains: a cysteine rich extracellular receptor, a trans-membrane region, and a cytoplasmic tail with a sequence known as the death domain (DD) (Schulze-Osthoff, 1998). The death domain is necessary for

binding an intercellular adaptor protein, Fas adapting death domain (FADD), which in turn can bind the apoptotic zymogen pro-caspase-8 (Chinnaiyan, 1995). Later studies showed that DR5 also bound FADD during caspase initiation (Schneider, 1997) , (Kuang, 2000).

Fas, a 319 amino acid protein, is an extensively studied death receptor and a component of the normal immune response (Itoh, 1991). The Fas/FasL system was first observed to induce apoptosis in activated T-lymphocytes (Owen-Schaub, 1992). The results explained why mice with a mutation in the Fas gene, known as *lpr* (lymphoproliferative) mice, had an increased tendency for auto-immune disease and helped to underline a normal function of Fas in the apoptosis of self-reacting T-cells (Watanabe-Fukunaga, 1992). Fas also mediates cell death following various injury models, notably therapies such as radiation. Removing or disabling Fas will often lead to tumor growth and cancer (Landowski, 1997), (Maeda, 1999), (Mullauer, 2001). Thus, Fas also protects cells from over-proliferation and its protein levels are positively linked to apoptosis in many cell types, including germ cells, and under many different conditions.

The Fas/FasL system is not the only important death receptor interaction in cell homeostasis. The TRAIL/DR5 system is also a key regulator of apoptosis. The apparent selective sensitivity of tumor cells to undergo apoptotic cell death by exposure to the cytokine TRAIL (tumor necrosis factor related apoptosis-inducing ligand) has led to considerable speculation as to its potential as a cancer therapeutic agent (Kimberley, 2004). In fact, TRAIL is currently being tested in human clinical trials for this purpose. (Duiker, 2006). TRAIL has also been reported to initiate apoptosis in certain non-transformed or primary cell types, including hepatocytes (Lawrence, 2001), (Mori, 2004). However, the underlying mechanisms that account for the sensitivity of cells to TRAIL-mediated apoptosis remain unsolved and is a topic of intense investigation. One hypothesis is that DR5 may act as a "substitute" system when other death receptors, such as Fas, are disabled. The TRAIL/DR5 system may be more active in different cell types, such as those with high proliferation rates, including the testis.

Each receptor-ligand tandem operates in an analogous way to promote cell death. Under conditions of apoptosis, Fas or DR5 may be present on the cell membrane initially as monomers but soon form homotrimers *via* association through their extracellular PLAD (pre-ligand binding association domain) region (Chan, 2000), (Siegel, 2000), (Daniels, 2005). Death receptor association is generally described as being trimeric, although the exact number is unknown and up to six receptors may associate at once to induce apoptosis (Holler, 2003). In the case of Fas, evidence shows that the minimum formation of homotrimers must occur or apoptosis is not able to properly initiate (Siegel, 2000), (Papoff, 1999). In this case, receptor ligands FasL and TRAIL probably must exist as trimers as well, producing a 3:3 stoichiometry upon interaction (Siegel, 2000). Thus, trimers of receptor and ligand must be present to activate apoptosis through the extrinsic pathway and trigger the first steps in the enzymatic cascade.

Receptor/ligand complexes stimulate the recruitment of the adapter protein FADD to the intracellular death domain of the receptor. FADD shares a N-terminal death domain complementary to the sequence in the C-terminal region of Fas or DR5. This complexation of receptor and adaptor attracts a zymogen called procaspases -8 or -10 and binding occurs between the death domain of FADD and the N-terminal death effector domain (DED) region of the caspase (Peter, 2003). The assembly of all the proteins—receptor, ligand, adaptor, and caspase—form a complex known as the death inducing signaling complex (DISC). The interaction of procaspases at the DISC platform allows for their proteolytic cleavage and activation of the caspase "cascade" required to initiate apoptosis (Muzio, 1996).

Death receptors associate on the membrane of the cell through their extracellular PLAD region prior to ligand binding, which then activates formation of the DISC (Siegel, 2000), (Algeciras-Schimnich, 2002), (Henkler, 2005). The formation of the DISC appears to promote aggregation of additional death receptor trimers on the cell surface, resulting in clustering and more potent apoptosis when the ligand triggers caspase cleavage (Siegel, 2000). The clustering sometimes occurs within a lipid raft, a cholesterol and sphingolipid rich region of

the cell membrane where many different type of signaling molecules reside (Scheel-Toellner, 2002). The generation of concentrated Fas trimer clusters on the membrane has been observed when fluorescently stained and observed microscopically as "Fas capping." (Belka, 1998), (Cremesti, 2001) Because DR5 also likely assembles higher order clusters on the cell membrane, caps theoretically can form after conditions of high expression as well. Recently, a report has identified increased DR5 association and clustering in lipid rafts with high levels of membrane ceramides (Martin, 2005).

ii) Caspases and the Apoptotic Pathways

A myriad of proteins are involved in the initiation, execution, and regulation of apoptosis. However, caspases are the proteins central to the process and are an absolute requirement for most types of cell death. The fundamental action of the caspase is to recognize and cleave target protein molecules after a specific aspartic acid residue. Cleavage will usually inactivate, but sometimes stimulate or even change the basic function of a given substrate. Apoptosis can occur independent of caspases, for example through the activity of apoptosis inducing factor (AIF) or lysosomal stress (Kroemer, 2005). However, caspase activation is observed in nearly all instances of traditional apoptosis.

Currently fourteen human caspase genes have been found. The proteins are expressed as zymogens called pro-caspases, which possess weak enzymatic activity. Pro-caspases can be organized based on the length of their N-terminal "prodomain" region, which also helps to determine the functional role of the protein. Caspases that possess a long prodomain (caspase-1, -2, -4, -5, -8, -9, -10, -11, -12) are able to interact with adaptor proteins to promote cell death and are designated initiator or apical caspases. As discussed in the previous section, TRAIL/DR5 or Fas/FasL triggering requires the adaptor protein FADD. FADD molecules possess death effector domains (DEDs) that bind a similar region on the N-terminal prodomain of the initiator pro-caspase. FADD also interacts with

the death domain (DD) region along the transmembrane tail of the death receptor (Chinnaiyan, 1996), (Chen, 2002). The combination of death receptor, adaptor protein, and pro-caspase is known collectively as the DISC, or death inducing signaling complex. The initiator caspase is most commonly caspase-8, but -10 has been shown to localize as part of the DISC for extrinsic apoptotic signaling as well (Kischkel, 2001).

The mechanism of initiator procaspase activation is still being studied. It is known that procaspase monomers have weak enzymatic activity (Muzio, 1997). Procaspases in the DISC are thought to be activated through cleavage by an adjacent procaspase. Although one initiator procaspase is inactive, a complex of many together will produce enough catalytic activity that each protein is cleaved and rearranged, forming the active conformation. The procaspases are arranged close to each other in order to achieve this autocatalytic processing, which is referred to as the *induced proximity model* (Muzio, 1998), (Salvesen, 1999). Once activated, the caspase forms a heterotetramer, which can interact with and cleave the effector caspases. Effectors represent the second group of proteases with short prodomains. Caspases -3, -6, -7, and comprise this group and execute the apoptotic program by cleaving important substrates in the cell, such as laminin, ICAD, and PARP. A refinement of this hypothesis, known as the *unified model*, argues that simple dimerization of procaspase-8 is sufficient to initiate activation (Boatright, 2003). However, both views agree that downstream activation of effector caspases is requisite for apoptosis.

Caspases have the ability to cleave after tetrapeptide motifs (denoted as P4-P3-P2-P1) when the P1 amino acid is an aspartic acid (Shi, 2002). Interestingly, caspases themselves are cleaved at a particular aspartic acid, resulting in the formation of large (~20 kD) and small (~10 kD) sub-units, a pro-domain, and a linker region (Cohen, 1997). The resulting heterotetrameric structure is considered the active form of the protein and this structural rearrangement is seen in both initiator and executor caspases.

In summary, the death receptor pathway always involves the initial engagement of a death receptor and its cognate ligand, activation of caspase-8, and the downstream cleavage of effector procaspase-3. The aggregation of death receptor-ligand complexes are thought to stimulate DISC formation by attracting the adaptor protein FADD and procaspase-8. In the most popular model, the close proximity of weakly proteolytic procaspase-8 molecules result in their autocleavage and the production of active caspase-8. Only then can caspase-3 be completely processed and facilitate the cleavage of cellular substrates that culminate in cell death. This process is mitigated by c-FLIP, an inhibitor that blocks caspase-8 activation in this process (**Fig. 1.4**). The function of c-FLIP will be fully discussed in the next section.

Apoptosis can also be generated through the intrinsic pathway (**Fig. 1.5**) Although this mechanism ultimately activates caspase-3, the upstream chain of events is much different and considerably more complex. The initiation of apoptosis utilizes the BCL-2 proteins Bak and Bax to create a channel in the outer membrane of the mitochondria organelle (Luo, 1998), (Korsmeyer, 2000). The pore introduced by Bax/Bak is referred to as the mitochondria permeability transmission pore (PTP), which accomplishes two events: the abolishment of the ordinary voltage potential across the mitochondrial inner membrane and the release of the pro-apoptotic molecule cytochrome c. The timing of the loss of membrane potential is controversial and may in fact occur post cytochrome c release (Gogvadze, 2006). Nevertheless, the PTP extends to the outer membrane, triggering the release of cytochrome c and its eventual integration as a component of the apoptosome. The apoptosome is a complex of ATP, caspase-9, cytocrome c, and an adapter protein called Apaf-1. It is well established now that this complex is responsible for the cleavage of procaspase-9 into active caspase-9. Ultimately, caspase-9 processes caspase-3 and propagates an apoptotic response leading to the death of the cell (Li, 1997), (Bao, 2007).

MEHP-mediated apoptosis is known to primarily stimulate the extrinsic pathway, and therefore the above description of intrinsic cell death has been

greatly simplified. (For a more in depth discussion of mitochondrial cell death, please refer to the following review, {Kroemer, 2003}) It should be noted that cross talk does occur between the two pathways. Under conditions of robust apoptotic activity in type I cells, caspase-8 activation may also act to cleave the BH3-only protein Bid into t-Bid (*truncated* Bid), acting with Bax/Bak to aid in the formation of the mitochondrial transmembrane pore and eventual apoptosis though the intrinsic pathway (Luo, 1998), (Scaffidi, 1999). Therefore, death receptors may at times induce the mitochondrial pathway if the activation of caspase-8 is high enough and Bid is expressed in the same cell. However, in type II cells (where caspase-8 activity is low), Bax is the principle activator of the mitochondrial pathway of cell death (Scaffidi, 1999), (Barnhart, 2003), (Rudner, 2005). The two pathways may be regarded as redundant in this manner, as the outcome of both pathways is activated caspase-3.

iii) Caspase inhibitors and apoptotic regulation

Given the critical role of caspases, tight regulation of their action is of the highest importance. Inappropriate activation of caspases is implicated in neurodegenerative disorders such as Alzheimer's Disease and amyotrophic lateral sclerosis, cardiac and liver dysfunctions, and infertility (Malhi, 2006), (Gervais, 1999), (Gustafsson, 2003). Often chemotherapeutic drugs and treatments engage the apoptotic machinery leading to the death of several tissues. To protect from unrestricted apoptosis, the cell has developed tools to combat the expression of caspases and prevent widespread death.

The most important inhibitor of the death receptor mediated pathway at the DISC is c-FLIP (FLICE-like inhibitor of protein, also commonly known as c-FLIP, c-FLAR, Flame, or usurpin), which was first identified in herpesvirus but is common to all known mammalian systems (Thome, 1997). c-FLIP mimics the structure of procaspase-8 and acts as a competitive inhibitor to that protein. This ability is due to the dual N-terminal DED (death effector domains) regions

homologous to those of caspase-8 (**Fig. 1.4**). However, the caspase-like region of c-FLIP is missing the catalytic cysteine region that would lead to the complete cleavage and activation of caspases (Scaffidi, 1999), (Krueger, 2001). Consequently, if expressed highly in the DISC instead of capsases, death receptor-ligand complexes will fail to induce apoptosis in most cases.

The mechanism appears to be one of competition between c-FLIP and caspase-8 for the adaptor FADD at the DISC and has been shown to be concentration dependent (Chang, 2002). High expression of c-FLIP can lead to partial reduction or complete inhibition of caspase-8 dependent apoptosis. The inhibition is believed to act by tying up the DED position on FADD and therefore inhibiting ongoing activation of procaspases present at the DISC. There is also some evidence that FLIP can physically bind procaspase-8 as determined by *in vitro* binding studies in HEK 293 cells (Hu, 1997). Thus, c-FLIP may have more than one substrate for which to block death receptor induced apoptosis.

c-FLIP can be detected as numerous mRNA transcripts but the protein is commonly expressed in only two forms, short (~28 kD) and long (~56 kD), although a lymphocyte-specific form has also been recently identified (Golks, 2005). The former is thought to be unambiguously anti-apoptotic and prevents cell death by blocking access of procaspase-8 to the DISC. Structural data reveals that c-FLIP$_S$ possesses a FADD binding region (death effector domain) homologous to procaspase-8 but lacks the catalytic domain altogether. Thus, the short isoform is not involved with biochemical processing at the DISC and functions to saturate procaspase-8 binding sites (Kirchhoff, 2000).

The long form is less well defined functionally, although most research has determined its function to be anti-apoptotic under normal physiological conditions (Sharp, 2005), (Tschopp, 1997). c-FLIP$_L$ contains a caspase-like prodomain and the catalytic domain, although lacking the cysteine-containing active site. However, at very low levels c-FLIP$_L$ may act to induce apoptosis *via* heterodimerization (Boatright, 2003). In the proximity model, homotrimeric procaspases are brought closely together to precipitate the apical caspase cascade.

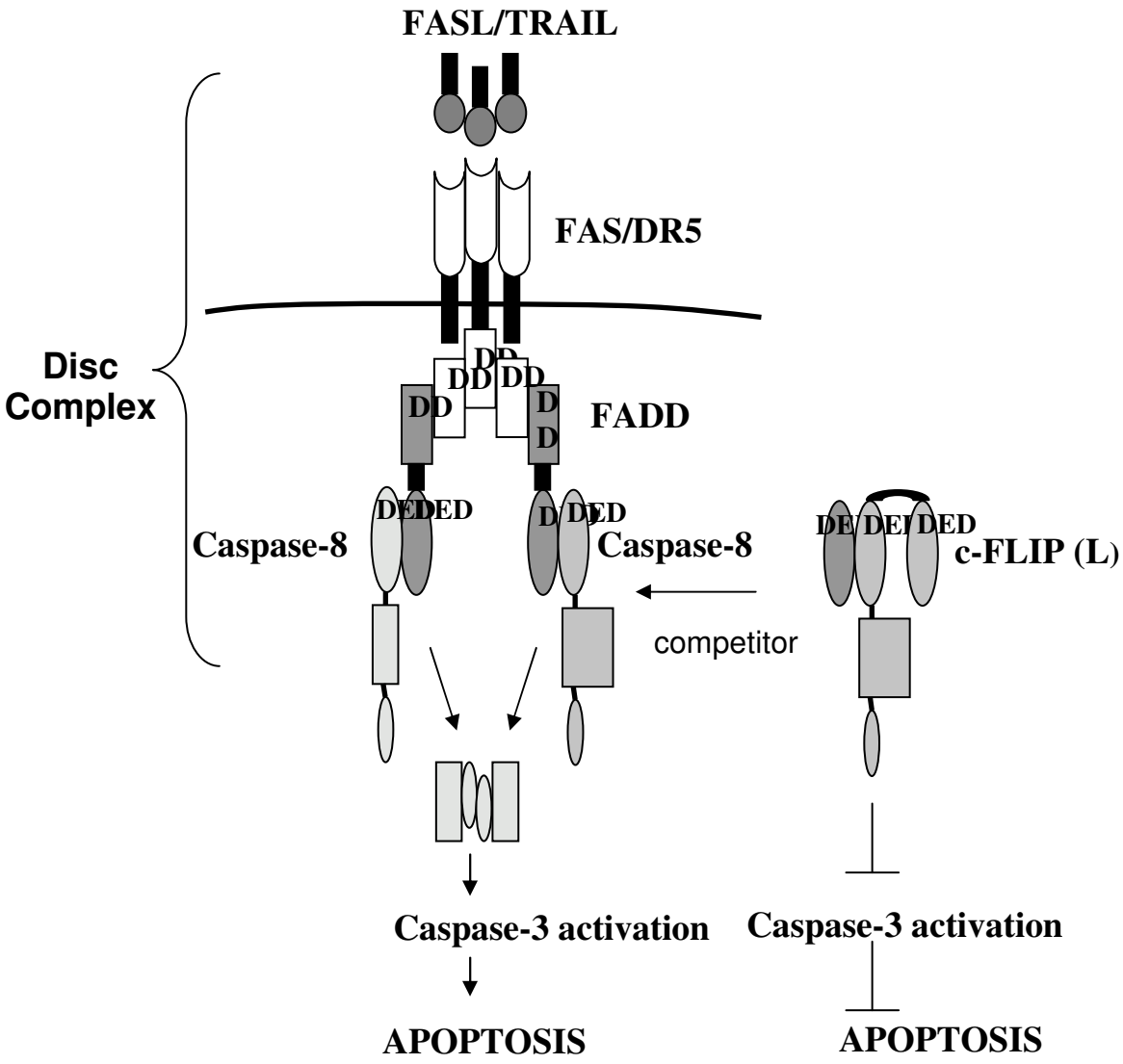

Fig. 1.4 *DISC formation and the mechanism of the extrinsic pathway.* Death receptor/ligand complexes recruit FADD and procaspase-8 to the membrane, which then activates downstream caspase-3 activation and cell death. The process can be blocked at the DISC by c-FLIP long or short (not shown).

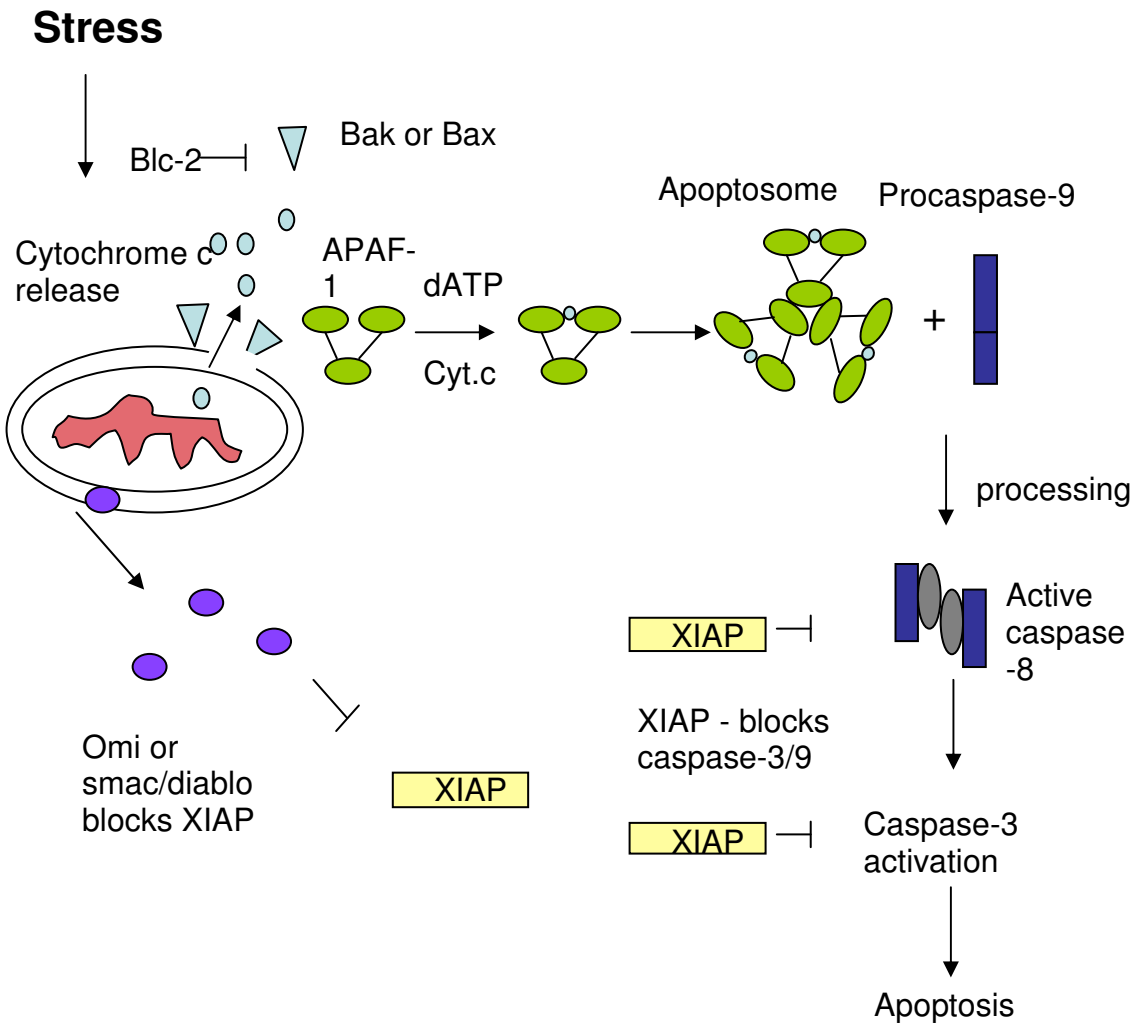

Fig. 1.5 *Apoptosome formation and mechanism of the Intrinsic Pathway.* Bak and Bax act to induce a pore in the outer membrane of the mitochondria, triggering cytochrome C release. The interaction of ATP, the adapter protein Apaf-1, and procaspase-9 constitutes the apoptomsome, leading to caspase-9 activation and cell death. XIAP functions to bind caspases and prevent their activation, thus is a major inhibitor of this process. Smac/Diablo can inhibit XIAP.

However, several reports have indicated that heterogeneous complexes of capase-8 and $FLIP_L$ may also trigger apoptosis under certain conditions or concentrations (Chang, 2002). This effect has been seen, for instance, in the augmentation of cell death in the T-cells (Dohrman, 2005). It is known that both c-FLIP and caspase-8 undergo proteolytic processing when mixed at the DISC, but in most reported cases the processed form of c-$FLIP_L$ (p43) inhibits further activation after an initial cleavage step. Therefore, the mechanism of c-$FLIP_L$ protein remains controversial.

Both c-FLIP short and long may be important to the survival of germ cells stressed by MEHP. The protein, particularly c-$FLIP_L$, has been detected in germ cells and protects them from death in response to apoptotic stimuli (Giampietri, 2003). c-$FLIP_L$ inhibits Fas and TRAIL mediated apoptosis in germ cells; the mechanism of action may partly be under control of p53, which has been suspected to increase death receptor levels to a point that c-FLIP can no longer provide protection (Chandrasekaran, 2005). The germ sub-type most effected by MEHP exposures is the spermatocyte, located in the adluminal compartment. Whether the c-FLIP is degraded to facilitate spermatocyte apoptosis is unknown. The regulation of this interaction may be important to the increased production of spermatozoa in toxicant-affected testis.

Another major class of caspase inhibitors are the inhibitor of apoptosis (IAP) family. As with c-FLIP, the discovery of the first IAP genes were derived from a virus species, bacculovirus (Crook, 1993). The protein was initially shown to impart protection against apoptosis in insects infected with the virus. However, various homologs exist across a variety of species, including *Drosophila*, *C. elegans*, yeast, and mammals. Mammals express c-IAP-1/2, survivin, and XIAP in a largely ubiquitous manner (Deveraux, 1999). Specialized transcripts, such as ILP-2 in the testis, have also been detected (Richter, 2001).

XIAP (X-chromosome-linked inhibitor of apoptosis protein) is the most studied of the IAP's and is considered the most potent inhibitor of cell death. In fact, the protein binds its target substrates more tightly at lower concentrations

than other members of the IAP family (Deveraux, 1999). Part of the reason is that unlike c-FLIP, the XIAP protein is able to effectively block caspase-3, the effector caspase for both the mitochondrial pathway and the death receptor pathway of apoptosis. In addition, XIAP can specifically bind and neutralize caspase-9, the initiator caspase for the mitochondrial pathway. XIAP regulatory proteins possesses three distinct regions called BIR's (baculoviral inhibitor of apoptosis repeats) that denote their binding specificity. The interaction between XIAP and caspases are thought to be largely mediated by the BIR regions and apoptosis is blocked by reduced access of the caspase to its substrate (Bratton, 2002). BIR-2 and a portion of a preceeding linking sequence bind caspase-3 and BIR-3 binds caspase-9.

Additionally, XIAP contains a c-terminal RING (really interesting new gene) domain and functions as an E3 ubiquitin ligase. E3 ligases are the final executors of ubiquitinylation, one of the most common types of post-translational protein modifications (Suzuki, 2001). E3 ligases are designed to add ubiquitin molecules onto the target proteins, which are then "marked" for cellular transport, endocytosis, or degradation by the proteasome. In this last instance, caspases and other cellular targets may be actually be removed by degradation in addition to being bound and sequestered by a given XIAP, although this theory remains speculative (Yang, 2000).

Interestingly, XIAP can also autoubiquitinylate. This self limiting step is another way in which the protein is designed to control overexpression. In addition, antagonists smac/diablo are released from the mitochondria and prevent inappropriate activity of XIAP by competing for the same binding sites that caspases occupy (Du, 2000), (Verhagen, 2000), (Srinivasula, 2000). The agonistic activity of smac/diablo is supplemented by Omi/htr2A, a mitochondrial protease that is believed to also compete for XIAP binding sites and possibly degrade the XIAP protein itself (Martins, 2002). However, the RING finger of XIAP is capable of ubiquitinylating these proteins as well (MacFarlane, 2002). Whether XIAP and its inhibitors are actively engaged following MEHP-induced apoptosis

is unknown; the toxicant has been shown to only weakly activate the intrinsic pathway and additional studies will be necessary to elucidate the role of XIAP. The process of ubiquitinylation and its role in regulating apoptosis will be further discussed in regards to c-FLIP, which may be sensitive to E3 ligases.

Another major group of apoptosis regulators are the BCL-2 family of proteins. Extensive research has been undertaken to define these modulators, which can be pro-apoptotic (Bak, Bax, Bad, t-BID, among others) or anti-apoptotic (bcl-xl, bcl-2, bcl-w). The key to their activity resides in the particular BH (BCL homology) domains. The apoptotic BCL-2 family members can be activated by DNA damage, radiation, and chemical stresses, and often operate in conjunction to form pores in the mitochondrial outer membrane. For example, t-BID formation promotes Bak oligimerization, which can then work in conjunction with Bax (Wei, 2000), (Korsmeyer, 2000). Together, they form the upstream component of the mitochondrial apoptotic pathway and aid in the release of cytochrome c following the destabilization of the organelle's membrane and ultimately the formation of the apoptosome. The survival protein BCL-2 inhibits this process by antagonizing Bax and preventing the destabilizing pore in the mitochondrial membrane. (For a comprehensive review, please refer to {Walensky, 2006})

1.3 Phthalates and Testicular Toxicity

A. DEHP and MEHP

MEHP (mono-2[ethylhexyl]-phthalate) is a major metabolite of plasticizing agent DEHP (di-2[ethylhexyl]-phthalate) that is found widely dispersed throughout the environment (Koch, 2006). Phthalates such as DEHP are used to soften plastics and lend to them a range of flexibility. The chemical is extremely widespread due to its use in polyvinylchloride (PVC) pipe as well as in plastic bags, food packs, and upholstery. In addition, phthalates are used in a

variety of solvents, oils, and adhesives. A more extensive list of phthalate containing products can be found in the comprehensive report written by the Center for Evaluation of Risks to Human Reproduction (Kavlock, 2002).

DEHP is not covalently bound to its plastic constituents and therefore loses contact with them over time. The primary route of exposure in humans is through ingestion, as DEHP concentrations contaminate drinking water and foodstuffs. Infants are often the most sensitive population, as bottles and pacifiers leach small levels of phthalate over time. Routine human consumption can be as high as 30 µg/kg/per day and 3.3 µg/kg/per day in infants , legitimizing concerns over exposure to phthalates (Albro, 1984).

DEHP is a di-ester acid that undergoes hydrolysis when metabolized and generates a mono-ester, MEHP, and several alcohol products (**Fig. 1.6**). However, MEHP is the most potent metabolic by-product in regards to testicular toxicity, probably because of its relatively high half-life in the testis. Ingestion of the DEHP compound produces different effects according to dose and species. In general, mammals experience dysfunction in the liver and reproductive tissues, including the testis. Rodent hepatocytes are particularly sensitive to DEHP metabolism and exhibit increased peroxisome proliferation (Gray, 1983). Occasionally MEHP has also been indicated in carcinogenesis, but only at extremely high and prolonged doses.

However, MEHP is of particular interest due to its effect on germ cell health and fertility. The compound has been shown in numerous studies to be associated with testicular apoptosis, morphological defects, and increased infertility (Gray, 1980), (Latini, 2006). These reasons, as well as its ubiquitous presence in the environment, justify the use of MEHP as a means to study the reproductive defects witnessed during spermatogenesis.

B. MEHP and Testicular Injury: A Model

MEHP is in appropriate choice for modeling testicular cell death for several reasons. Physiologically, MEHP exposure induces testicular abnormalities such as lower testicular weights, loss of germ cells, vacuolization, and hormone secretion (Richburg, 2002), (Richburg, 2003). However, MEHP is also thought to specifically target the Sertoli cell. In doing so, the health of germ cells dependent on Sertoli cell health will be indirectly damaged as a result; MEHP toxicity is known to interfere with proper growth factor release, disruption of hormonal balances, and the induction of pro-apoptotic ligands specific for germ cell membranes (Boekelheide, 2005). However, Sertoli cells rarely die as a result of direct MEHP toxicity and are known to remain robust in response to even very high toxic exposures (Richburg, 2002). Hence, MEHP causes a cellular "stress" or damage that transiently effects Sertoli cell homeostasis. The loss of the cell's stability in turn results in lower germ cell support capacity (**Fig. 1.7**). Phthalates have been implicated in numerous testicular and spermatogenic abnormalities: cryptorchidism (failure of the testes to descend), testicular cancer, and low sperm count. Several symptoms combined is referred to as testicular dysgenesis (TDS). TDS has been linked with failure of spermatogenesis, followed by reduced mature sperm numbers and infertility (Sharpe, 2001), (Fisher, 2004).

DEHP is reduced to MEHP *via* hydrolysis in the stomach and intestines in mammals (Albro, 1989). A possible reason why MEHP maintains such high toxicity in the seminiferous tubules can be attributed to its lipophillic character, which extends the duration the toxicant can spend in the testis. However, the sensitivity of the toxicant is variable among animals, with rodents the most sensitive and monkeys the least. Rodents appear to be unable to further metabolize MEHP, leading to their long-term presence in the testis, commonly a two day half-life (Oishi, 1982). Most humans actively glucoronidate MEHP to water soluble by-products and are able to excrete the toxicant. An important exception are infants, which lack the ability to biotransform MEHP and are

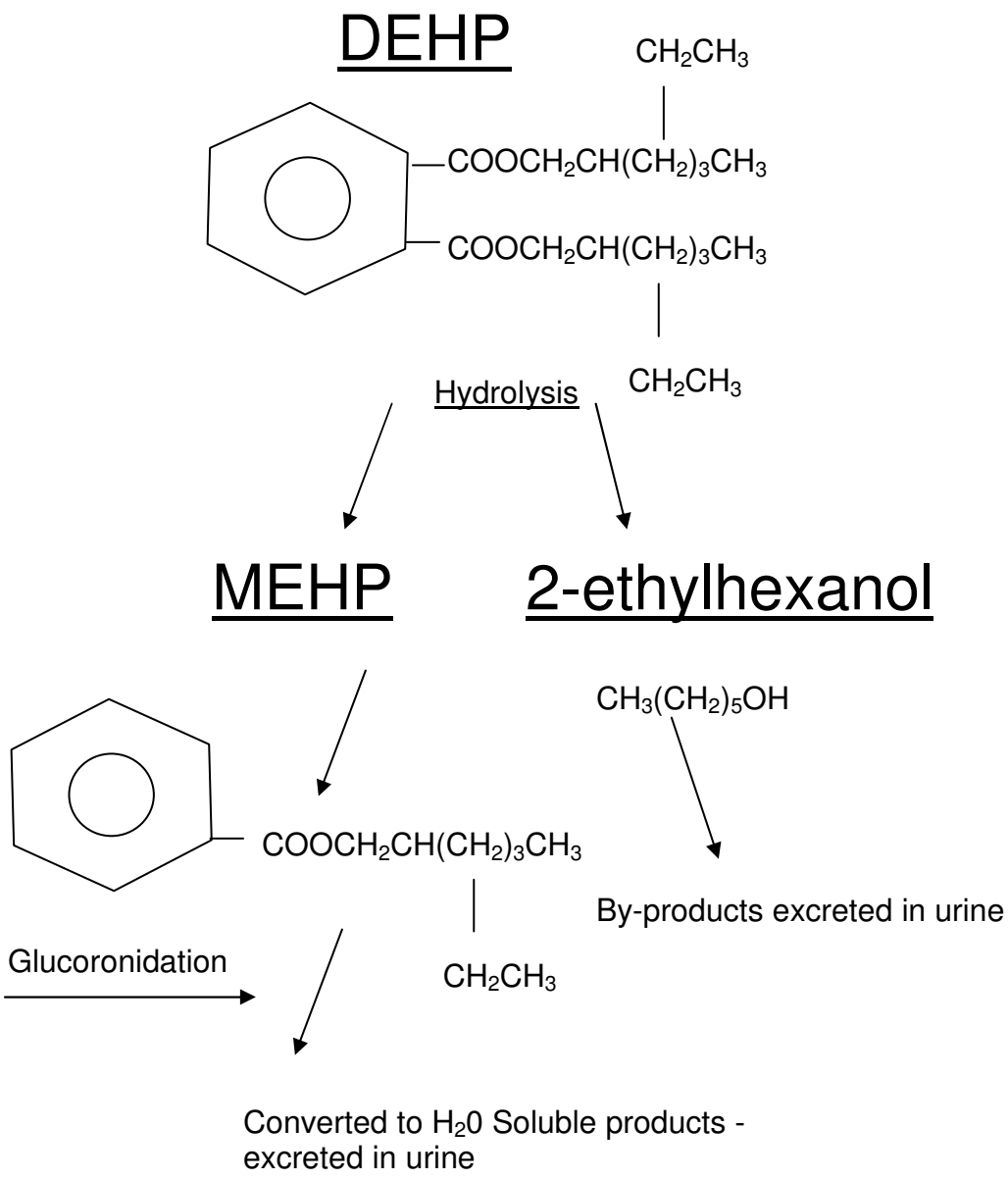

Fig. 1.7 DEHP Products. DEHP is metabolized into two major products via hydrolysis, MEHP and 2–ethylhexyl phthalate. MEHP is lipophillic, so the compound is glucoronidized in the liver to make it more water soluble, allowing excretion through urination. Infants under the age of four months do not have this ability, often leading to MEHP-mediated developmental defects and testicular dysgenesis.

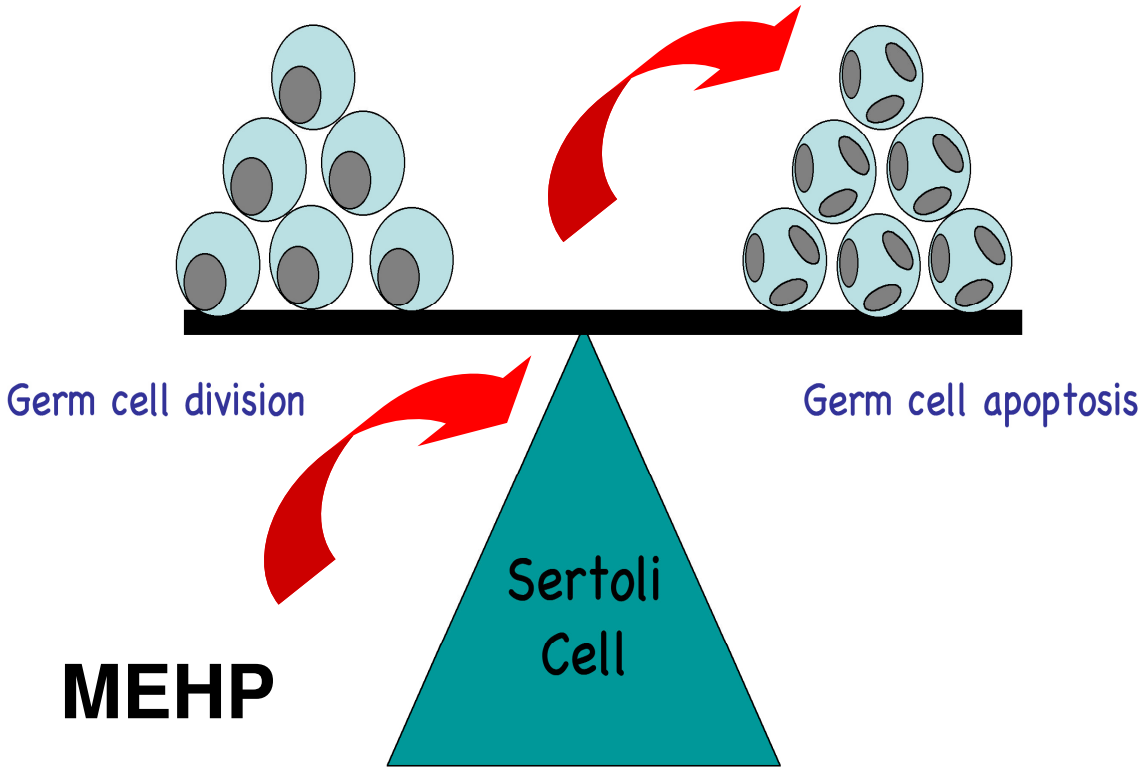

Fig. 1.7. *MEHP and germ cell homeostasis*. Proliferation or apoptosis of germ cells is determined by the status of the Sertoli cell. MEHP treatments push the balance toward cell death.

environmentally exposed to the compound *via* bottled formula and pacifiers (Lottrup, 2006).

Acute doses of 1g/kg by oral gavage are sufficient to induce significant morphological changes in Sertoli cells as early as three hours. Although significantly higher than ordinary human exposures, the dose is useful in determining gross changes in effected tissues and to study its mechanism of action. Vacuolization of the seminiferous tubule is an early indicator of germ cell damage and later stages exhibit Sertoli cell retraction ("flattening") along the basement membrane and significant apoptosis in spermatocytes and spermatids.

Changes in Sertoli cell morphology suggest that it may be the direct target of MEHP. For example, the Sertoli cell intermediate filament vimentin is affected by treatments, resulting in a structural derangement that leads to germ cell death (Richburg, 1996). Biochemical products such as lactate and pyruvate are also altered when MEHP is added to Sertoli cell cultures, effecting their energy producing and secretory functions, while germ cells appear to be uneffected (Williams, 1989). Despite these effects, Sertoli cells may stop proliferating but rarely die (Boekelheide, 1991). Developmental data has shown that chronic exposure to MEHP slows their initial proliferation of Sertoli cells and reduces their number per tubule (Dostal, 1988). However, damage to Sertoli cells represents a particularly important factor in the death of germ cells above the tight junctions that separate the basal and adluminal compartments. Extented MEHP exposures can reduce or damage Sertoli cell populations enough to eventually deplete the tubule of all but the earliest germ cell populations (such as stem spermatogonia), underlining the importance of Sertoli cell participation in spermatogenesis.

Investigations into the mechanism of MEHP toxicity have shown a myriad of testicular alterations post exposure. Zinc deprivation, competition for FSH receptors, and interference with steroid production have all been put forth as possible reasons for toxicity (Boekelheide, 2005). MEHP in adult rodents have

shown the highest level of testicular damage in the stages that FSH is the most highly expressed, suggesting a possible molecular target for toxicant within the Sertoli cell (Grasso, 1993). The PPAR (peroxisome proliferation-activated receptors) family of receptors has also been considered as a target, given the ability of MEHP to induce peroxisome formation in the liver (Braissant, 1998) However, no one hypothesis has produced a definitive answer as to what the actual mechanism may be.

Another possibility is related directly to the physical interaction of germ cells and Sertoli cells. As aforementioned, Sertoli cells are designed to provide support and organization to germ cell populations. Lacking this proper architecture, spermatogenesis is impaired. Physical detachment of Sertoli cells from germ cells in co-cultures can initiate cell death at lower levels than required for morphological changes (Gray, 1984). Various sources have compiled evidence that the disruption between these two cell types will lead to the apoptotic mechanism (Dirami, 1995), (Sawhney, 2005).

To date, no papers have definitively shown whether MEHP acts though the extrinsic or intrinsic apoptotic pathway. Some light background levels of activated caspase-9 can be detected post-MEHP treatment, suggesting the protein is involved. However, evidence strongly favors the extrinsic pathway, particularly in regards to the production of death ligands and their receptors in cells that have been damaged or "stressed" by the toxicant. One further note concerns the distinction between whether germ cells preferentially undergo extrinsic (type I) or intrinsic (type II) apoptosis. Type I cells respond to the addition of death receptors by formation of the DISC complex and activation of large quantities of caspase-8. Type II cells activate only enough caspase-8 to cleave Bid into t-Bid and to reinforce the cleavage of procaspase-3 (Scaffidi, 1999).

The classification of germ cells as either type I or type II is ambiguous and controversial. As a heterogenious population in the testis, each germ cell type may in fact have a different classification. Spermatogonia, one of the earliest germ cell sub-types, is relatively insensitive to death receptor stimulation and requires the

BCL-2 protein Bax to undergo apoptosis (Russell, 2002), (Sugiyama, 2001). In contrast, spermatocytes appear to be very sensitive to death receptors while the intrinsic pathway remain unengaged (Lizama, 2006), (Giammona, 2002). However, the apoptotic strategy of these cells remains unsolved and which pathway involved may in fact be a function of the toxicant used. For example, the chemotherapeutic drug cisplatin generates mitochondrial apoptosis in both spermatocytes and spermatogonia (Zhang, 2001). To this point, MEHP has been seen to strongly induce the death receptor pathway in spermatocytes, thus a type I response is observed.

The engagement of apoptotic signaling programs in response to Sertoli cell stress is one of the principle reasons for employing MEHP in the laboratory. The Sertoli cell plays an essential role in controlling germ cell numbers and any damage can produce apoptosis. This induction of cell death has been suggested to drive germ cell eradication following environmental exposures of phthalates and therefore promote infertility in mammalian males. How this is accomplished is still undetermined and subject to considerable scrutiny. MEHP challenges were developed to elucidate what apoptotic pathways are induced following germ cell death and to better understand the mechanisms of cell death in testicular tissues.

1.4 MEHP-mediated Signal Transduction in Germ Cells

MEHP has been used for industrial purposes for more than sixty years. However, only in the last twenty years has study of the toxicant been seriously investigated in the reproductive tissues. Because MEHP is both ubiquitous in the environment and a known testicular toxicant, the study of its mechanism is special significance in the field of reproduction. Low fertility is the most common physiological outcome of both chronic and acute exposure, but the pathway leading to these outcomes remains unknown. A clue to the mechanism of infertility lies in the apoptosis observed in germ cells following MEHP exposure. Expression of certain proteins have been shown historically to modulate the

fundamental balance between apoptotic and survival pathways. Elucidating which of these factors are most involved has been the aim of much research in our and other laboratories.

A. The Influence of p53 on Germ Cell Apoptosis

The regulatory gene and transcription factor p53 may be the most important single agent in the development of tumor production and cancer. In fact, data compiled suggests that p53 alterations are present in 50% or more of all human cancers (Lavin, 2006). The correlation with cancer is not surprising given the wide range of processes mediated by the expression of the gene, from transcription of genes in the nucleus to direct binding of cellular substrates. Mutation, over-expression, or deletion of the gene and its protein product result in the dysfunction of a wide range of critical cellular processes, including DNA repair, cell cycle control, stress signaling, and apoptosis (Bargonetti, 2002).

The structure of p53 reveals insights as to why the protein plays such a major role in the maintenance of homeostasis of the cell. The gene encodes a 393 amino acid protein with three major regions: a transactivation domain (TA), a central binding domain (CBD), and a regulatory domain (REG). (**Fig. 1.8**) Each domain confers a different role important in establishing the functionality of p53. The CBD is used to associate with specific DNA sequences for transcription of target genes and recognizes the inverted repeat sequence PuPuPuC(A/T)(A/T)GPyPyPy. This region composes the largest segment of the protein (a.a. 100-300) and also exhibits the highest level of loss of function mutations (Resnick-Silverman, 2006). The transactivation region is involved in the binding of transcription machinery and the co-factors required for specific gene targeting and transcription (Reed, 1993). The c-terminal regulatory region possesses a nuclear localization sequence that allows the protein to shuttle from the cytoplasm into the nucleus as well as a tetramerization domain required for protein-protein interactions among other functions (Chene, 2001) .

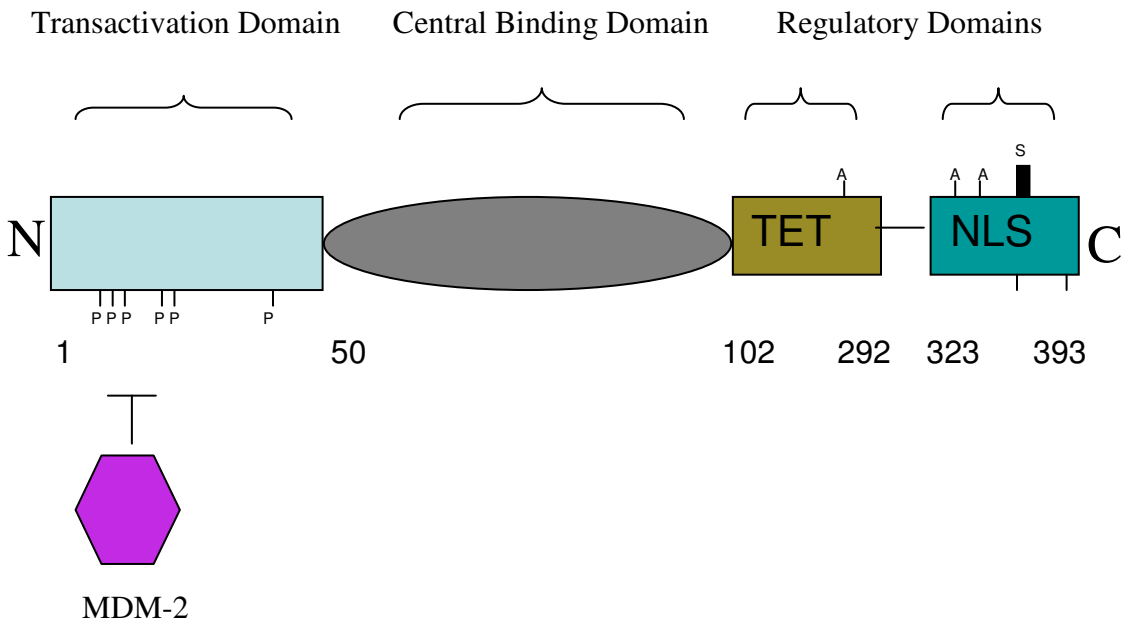

Fig. 1.8. p53 structure. Contains three major domains: 1) transactivation domain, responsible for binding transcription machinery. This domain's specificity is controlled by various phosphorylation sites. For example, phospho-serine 15 and 20 have been shown to prevent binding of p53 inhibitor, MDM-2. 2) The DNA binding domain, which controls the actual binding of p53 to its DNA substrates and is known to frequently contain mutations. 3) The regulatory region contains a tetramerization domain that assists in various protein-protein functions and a nuclear localization domain. Like the transactivation region, these c-terminal domains are regulated through various post-transcriptional modifications, including phosphorylation, acetylation, and sumoylation.

Because of the variety of roles that p53 potentially plays in the cell, strict regulation is a necessity. All domains of the p53 protein possess at least one site of post-translational modification, such as for phosphorylation, acetylation, sumoylation, or ubiquitinylation. The p53 protein has a short half-life and is kept at low levels under normal conditions unless a cellular stress is detected. The double minute 2 protein (MDM-2) is probably the best known regulator of p53 activity (Haupt, 1997), (Momand, 1992). This protein is capable of mediating ubiquitin addition to substrate proteins with a RING ligase and in conjunction with its constituent E2 conjugating enzyme (Oliner, 1992). MDM-2 binds the N-terminal transactivation domain of p53 and adds a string of ubiquitins to "tag" the protein for proteolytic degradation. (Barak, 1993), (Haupt, 1997). Phosphorylation at specific serines present in this region inhibit the action of MDM-2 and stabilize p53 (Shieh, 1997). These modifications vary by cellular stress (such as heat shock versus ionizing radiation), although phosphorylation of p53 serines 15 and 20 (serine-18 in mice) are known to specifically prevent MDM2 binding (Shieh, 1997). Phosphorylation and acetylation in the C-terminal regulatory domain may also inhibit degradation of p53 or possibly effect its nuclear import/export signals. As with phosphorylation, it is likely that the particular type of stress plays a role in which modifications are made and when.

Various reports have identified the activity of p53 in the initiation of intrinsic apoptosis by influencing the expression or activation of the pro-apoptotic bcl-2 family proteins, such as Bax (Miyashita, 1994), (Miyashita, 1995). Bax is one of several pro-apoptotic proteins able to localize to the mitochondria and cooperate in the formation of a permeabilization pore in the organelle's outer membrane, allowing cytochrome c release. A pair of proteins, noxa and puma, may assist in the activity and localization of bax to the mitochondria (Haupt, 2003). The genes for all three proteins contain p53 response elements and are transcribed upon UV or genotoxic stress, supporting an important role in response to DNA damage. Demonstrating the complicated nature of apoptotic regulation in

cells, p53-dependent apoptosis can also be regulated by the over-expression of anti-apoptotic protein BCL-2 in virutally all cell lines (Wang, 1993).

The regulation of p53, tumorigenesis, and apoptosis are intrinsically linked. Radiation, DNA damaging agents such as etoposide, and hydrogen peroxide are examples of genotoxic stress agents that promote cell cycle catastrophe and promote apoptosis. p53 is engaged and stabilized under these conditions, often through phosphorylation of the transactivation domain, leading to the transcription of genes in the apoptotic pathway. The mutation of p53 can prevent transactivation of certain pro-death genes charged with preventing unchecked growth. As a result, cell cycle checkpoints are ignored, leading to tumorigenesis in the effected tissues. To abrogate this effect, apoptosis is employed to limit uncontrolled, or metastatic, cell growth. Bax, Noxa, Puma, Bid, and Apaf-1 are examples of proteins transcribed by p53 in response to genotoxic stress (Schuler, 2001). Many of these gene products are active in the mitochondrial-mediated pathway. p53 activation/stabilization in the nucleus will trigger expression of these proteins, leading to destabilization of the outer mitochondrial membrane, the release of cytochrome C, and formation of the apoptosome.

However, p53 is not only involved in cancer or DNA-damage scenarios. Non-genotoxic stresses can stabilize the protein as well, including toxicants and certain chemotherapeutic agents, leading to the up-regulation of pro-death receptors in the extrinsic pathway such as DR5 and Fas. Previous research has established that both death receptors contain p53 response elements that induce their transcription (Owen-Schaub, 1995), (Wu, 1997). However, the conditions specific for cell death are variable according to cell type and injury model, as are death receptor/p53 relationships. For example, the thymocytes of *gld* (FasL mutant) mice undergo p53-mediated apoptosis despite lacking a fully functional Fas/FasL system (O'Connor, 2000) In contrast, ionizing radiation increases p53 stability and leads to higher levels of Fas-dependent apoptosis in germ cells (Embree-Ku, 2002). Heat shock applied to whole testis also engages the Fas-FasL

system and further established a relationship with p53 activity and the death receptor expression (Miura, 2002). Germ cells express high levels of both death receptors after MEHP-mediated cellular stress and that this may be the result of p53 activation.

There is some evidence suggesting a mechanism by which p53 aids the shuttling of Fas from the Golgi to the membrane (Bennet, 1998). A recent study in our lab showed that p53 may influence the levels of both Fas and DR5 by increasing protein movement to the cell membrane. In this model, p53 deficient animals showed reduced death receptor levels on germ cell plasma membrane surfaces and a predicted decrease in apoptotic sensitivity. (Chandrasekaran, 2005) The study suggests that Fas transcription is not the only method to elevate Fas membrane concentrations and that p53 stabilization could be involved in protein trafficking as well.

The multi-functional character of p53 is one of the initial aims of the following dissertation, which seeks to understand the relationship between pro-apoptotic proteins (particularly death receptors) and their downstream modulators (c-FLIP). The regulation and function of p53 is very complex and is involved in numerous cellular pathways. The supposition here is that p53 can play a role in sensitizing germ cells to death after MEHP-mediated Sertoli cell injury. Whether the signal is initiated by the withdrawal of a growth factor or another messenger is unknown. Nevertheless, p53 is believed to be a critical component to germ cell death and appears to effect the expression of pro-death elements following cellular stress.

B. Death Receptor Expression

Sertoli cells have been shown to release death ligands in addition to growth factors and previous investigations have revealed that the death receptor mediated pathway may be important in response to MEHP exposure. Lee *et al* showed that MEHP administration in adult rats led to an increased expression of

FasL from the Sertoli cell and a concomitant increase in Fas receptor in the germ cells. Therefore, the germ cells supported by adjacent effected Sertoli cells were subject to apoptosis (Lee, 1999) Another experiment tested the apoptotic levels of germ cells following MEHP using FasL mutant mice (*gld*). *Gld* mice have a mutation in the c-terminal region of the FasL protein, preventing efficient binding to its substrate, Fas (Takahashi, 1994). The reduced sensitivity to MEHP suggests that the toxicant preferentially acts through the death receptor mediated pathway and the experimental result confirms that the Fas/FasL system plays a role in germ cell apoptosis. However, it also illustrates that simple abrogation of one apoptotic protein is not enough to completely inhibit the apoptotic pathway (Richburg, 2000). In fact, *gld* mice exhibit only moderate changes in testicular morphology and are fertile. Further data suggested that other death receptors, such as DR5 (TRAIL R2), were also expressed after MEHP challenges (Giammona, 2002). The essentially normal phenotype of the mice would imply the possibility of a redundant death receptor system such as the TRAIL/DR5 tandem.

The TRAIL/DR5 system is particularly interesting in studies of the extrinsic pathway. Like Fas, DR5 is a TNF family member and type II transmembrane protein able to stimulate apoptosis *via* interaction with a death receptor (el-Deiry, 1998). The active receptor is DR4 or DR5 in most mammalian systems although there also exists two truncated "decoy" forms of DR5 that are expressed in certain tissues but are unable to induce apoptosis due to a truncated cytoplasmic tail region (Sheridan, 1997). Decoy receptors have been suggested as a way for the immune system to differentiate between normal and over-proliferative cell types (Kim, 2000). The cytoplasmic region of DR5 receptors possess two death domains that are similar in homology to that of Fas and function to recruit the FADD adaptor molecule (LeBlanc, 2001). TRAIL is also a TNF-alpha family transmembrane protein and serves as its cognate ligand for DR4/5. Like Fas, DR5 can form trimers as well as be solubilized by cleavage from the cell surface (Wu, 1997). The complex of TRAIL with active DR5

triggers apoptosis in a manner homologous to Fas/FasL and acts to initiate the extrinsic caspase cascade required for cell death.

However, unlike Fas, TRAIL is usually active only in tumor or transformed cells. This affinity for cancer cells *versus* normal healthy cells have made the protein a promising aid in combating tumorigenesis, although clinical trials have had mixed results (Duiker, 2006), (Gajewski, 2007). The TRAIL/DR5 system is not commonly expressed in physiologically normal cells but there are a few notable exceptions, such as in hepatocytes and possibly in the reproductive tissues (Mori, 2004). Protein and mRNA levels of TRAIL and DR5 are highly expressed in both human and mouse testis. TRAIL is especially high in the Sertoli cells and Leydig cells, whereas the expression of the death receptors appear to be variable among germ cell type to age specificities (Grataroli, 2002). There is evidence that MEHP exposure leads to an increase in DR5 levels in *gld* mice, presumably to replace the reduced function of FasL in facilitating apoptosis.

The particular distribution of death receptors and their ligands is an important question in how germ cells are regulated using programmed cell death. The receptor is an absolute requirement for cells to be sensitive to the extrinsic pathway of apoptosis. In rare cases, the ligand is released from an adjacent cell, although some cell types can produce their own autocrine death ligands. Cytotoxic T-cells are an example of a cell that expresses both a death receptor and its cognate ligand (Dhein, 1995). However, other cells are unresponsive to the presence of death receptors. Spermatogonia, for example, express DR5 but are impervious to apoptosis in the presence of TRAIL (Lizama, 2007). Spermatocytes, on the other hand, are shown to be very sensitive to both FasL and TRAIL expression. Because of the importance of death receptor-mediated apoptosis following MEHP exposure, an understanding of germ cell specificity and timing is critical.

However, the nature of death receptor activation in germ cells post-MEHP remains an unanswered question. Direct injury to Sertoli cells has been reported, concomitant with death ligand increases, but MEHP does not alter the germ cells

themselves in a specific and immediate way. Instead, the injury imposed upon the Sertoli cell "stresses" germ cell populations, whether by retraction of a growth factor or some another signal, leading to higher levels of death receptors on the their cell membranes. An important signal in germ cell apoptosis may be the activation of p53, followed by the transcription, trafficking and clustering of death receptors. The mechanisms downstream of this stress and the specific cells in which these mechanisms act composes the bulk of this dissertation.

C. The Role of Ubiquitinylation in FLIP Regulation

The activity that occurs at the DISC will determine whether of not apoptosis occurs through the death receptor mediated pathway. As discussed in the previous section, the accumulation of death receptors on the cell membrane are thought to promote the assembly of the DISC components at the membrane and initiate the apoptotic cascade. Like other steps in the apoptotic pathway, a fundamental balance between death and survival proteins is observed. At the DISC, once formed, that balance is influenced by the ratio of procaspase-8 molecules and their direct inhibitor, c-FLIP. Which protein is found in the highest concentration at the DISC is thought to an important determinate of whether apoptosis proceeds from this point or is blocked.

The place that c-FLIP occupies in apoptosis is sometimes uncertain. The short form (c-$FLIP_s$) is thought to be unambiguously anti-apoptotic and has been shown to completely block caspase-8 cleavage at the DISC (Bin, 2002), (Krueger, 2001). The mechanism is thought to be mediated by the direct binding of the molecule to the DED of FADD in a competitive saturation of caspase-8 binding sites. $FLIP_L$ is believed to interact in much the same way, although the protein contains a caspase-like pro-domain that can be cleaved at Asp-341 (Thome, 2001). This enables c-$FLIP_L$ to be processed once in a fashion similar to procaspase-8, although the processed form is thought to remain at the DISC as an effective block to apoptosis. However, the ability for procaspase-8 and $FLIP_L$ to

form heterodimers at the DISC has produced conflict within the literature. The possibility that heterodimers act to increase procaspase-8 cleavage has been reported and seems to be concentration dependent. There is also evidence that $FLIP_L$ – mediated procaspase cleavage may increase apoptosis *via* the processing of other caspase substrates in the DISC, such as receptor interacting kinase (RIP) (Boatright, 2004), (Micheau, 2002).

In the testis, c-FLIP levels have been identified with cell survival (Giampietri, 2003). However, relatively little research has been completed on the role of FLIP in germ cell populations and few have examined its regulation. A seminal paper by Fukazsawa *et al* suggested that c-FLIP may be targeted for the degradation in cancer cells when proteasome inhibitors blocked a decrease in the protein following p53 dependent apoptosis (Fukazawa, 2001). Both proteins show changes in concentration after MEHP treatment in the testis. The presence of p53 and c-FLIP within the same regulatory pathway suggests a link between the two in germ cell apoptosis.

Following the initial Fukazawa paper, ubiquitinylation has been associated with the regulation of the c-FLIP proteins. Both c-$FLIP_L$ and c-$FLIP_s$ undergo quick turn-over in the cell (four hours for $FLIP_s$, if treated with CHX); particularly $FLIP_s$, which has shown to be susceptible to the ubiquitin pathway due to target recognition residues on its c-terminus (Golks, 2005), (Poukkula, 2005). To date, no such region has been identified on the long form. The change in relative half-life between the two isomers is among the interesting differences between two proteins that share extensive homology. The c-$FLIP_L$ and c-$FLIP_s$ are known to act at the DISC but new evidence suggests that the long form may possess regulatory functions that have yet to be elucidated. For example, c-$FLIP_L$ has shown an unexpected activity in lymphocyte proliferation (Lens, 2002). The last aim of this dissertation is to shed light upon the role that c-FLIP plays in germ cells and to understand how the protein is ultimately regulated. Since c-FLIP potentially holds the key to whether a germ cell lives or dies, the key to elucidating its role in infertility is an important one.

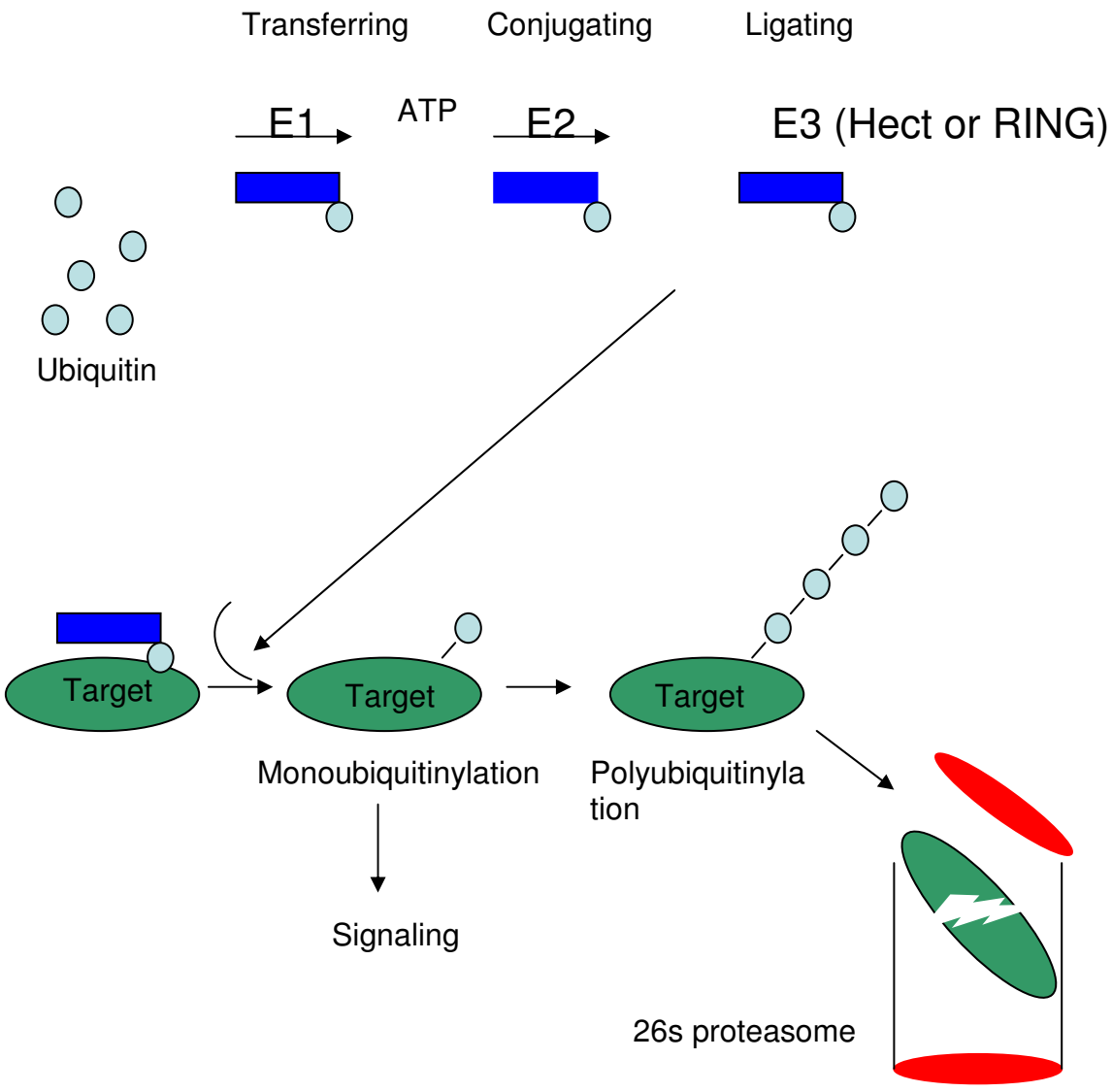

Fig. 1.7 *The Ubiquitin-Proteasome System*. Loose ubiquitin is covalently bound to the E1 enzyme via ATP and transferred through the intermediate E2 before target specificity is determined by the E3 ligase. Whether the target is fated for the proteasome or another cellular function is determined by how many ubiquitins are added.

Ubiquitinylation is the process of post-transcriptionally modifying proteins targeted for a cellular process. This is accomplished by covalently adding small (~8 kD) proteins called ubiquitin onto exposed lysine moieties in a given protein. The mechanism of individual ubiquitin molecule transfer occurs *via* three distinct enzymes: E1 (activating), E2 (conjugating), and the E3 (ligase) (**Fig. 1.7**). The activation process is ATP-dependent and biochemically requires the formation of a thiol ester between the c-terminal glycine of ubiquitin to a substrate lysine (Hochstrasser, 1992). Conjugation is an intermediate step, mediated by an E2 ligase, which involves passing the activated ubiquitin to an E3 ligase, for direct attachment to the targeted protein (Hershko, 1992). The E1 enzyme is a highly conserved enzyme and usually exists as only one or two varieties among individual organisms whereas E2 and E3 can be found in more varieties. The E3 enzyme is divided into two families (HECT and RING) but both aid in physically ligating ubiquitin molecules onto lysine residues. The RING ligase varies somewhat from HECT domains because it works together with the E2 conjugating enzyme to ubiquitinylate target substrates (Ingham, 2004). The HECT ligase directly attaches ubiquitin unaided.

The ultimate target of the ubiquitin labeled protein is the proteasome, located in the cytosol of all cells. The 26s proteasome is multi-subunit complex (~2000 kD) that cleaves apart ubiquitin-protein conjugates with the use of ATP. Inhibition of the proteasome is a component of some chemotherapies and is commonly accomplished with compounds such as lactacystin, which blocks chemotrypsin activity (Kornitzer, 2000). However, in many cases proteasome inhibition effects proper clearing of other molecules, and addition to germ cell cultures leads to massive cell death.

The function of ubiquitin relies on two factors: 1) which lysine is thilolated on the protein and 2) how many ubiquitins are strung together through that thiolation. If four or more ubiquitins are added to any one lysine (usually K48) then that protein is targeted to the proteasome for degradation. This process is referred to as poly-ubiquitinylation. However, thiolation of K29 or K63 are

known to support other fates for the cell. For instance, DNA damage repair occurs when K63 is ubiquitinylated (Pickart, 2001), (Hofmann, 2001). Sometimes proteins are also mono-ubiquitinylated on different lysines, resulting in changes in protein trafficking, endocytosis, vesicle formation, and other functions (Haglund, 2003).

An increasing amount of evidence suggests that c-FLIP levels are maintained through ubiquitinylation (Fukazawa, 2001), (Poukkula, 2005), (Chanvorachote, 2005). However, the E3 ligase responsible for its degradation is unspecified. One possibility is the HECT E3 ligase Itch, which is a component of T-cell cytotoxicity and regulation. The Itch protein has been implicated in targeting c-FLIP for degradation in liver cells (Chang, 2006). Further studies of Itch may help unravel the role of c-FLIP in germ cell apoptosis.

Although the ubiquitin pathway has been extensively described, little has been published in regard to its function in germ cell death or survival. However, given the importance of FLIP to controlling the death receptor-mediated pathway, an evaluation of its ability to undergo post-translational regulation is germane to our research. In addition, death receptors as well as germ cell death may be under the control of the p53 protein. In fact, experiments with p53-/- mice have shown a reduced amount of cell death and Fas expression, with a correlative increase in c-FLIP levels (Chandrasekaran, 2005). However, many components of germ cell specificity, c-FLIP processing at the DISC, and FLIP regulation are all currently unresolved. By using the stress-inducing environmental toxicant MEHP, these questions can be answered. In doing so, the mechanism of germ cell death will allow greater insights into how the testis respond to injury.

1.5 Aims of the Dissertation

Male infertility is a widespread physiological disorder that effects men of diverse backgrounds, ages, and ethnicities. Infertility is usually described as an inability to properly produce a threshold level of mature sperm (>20 million/ml),

suggesting a problem with the normal function of spermatogenesis. The cellular mechanisms that disrupt spermatogenesis and result in infertility are only partly understood. However, there is increasing evidence that apoptotic regulation is an important contributor to the proper maintenance of germ cells within the spermatogenic process. Furthermore, the homeostasis of the Sertoli cell is thought to be at the heart of relative germ cell number and maturity.

MEHP is an established Sertoli cell damaging agent and a common environmental toxicant. The use of MEHP allows for the analysis of how Sertoli cell damage induces germ cell stress and ensuing apoptosis. The approach is logical based on the known findings that the Sertoli cell sets the maximum number of germ cells to be supported in the testis. Prior research in our lab and others have determined that Sertoli cell damage shifts the balance from germ cell proliferation to death, reducing mature sperm within the testis, and potentially advancing lower fertility.

We posit here that MEHP may contribute to testicular dysgenesis by up-regulating apoptotic factors expressed in germ cells, particularly those above the Sertoli cell tight junctions. These are the meiotic germ cells that primarily rely on the support of the Sertoli cell and thus would be the most effected by a toxicant challenge. Significant reduction of this segment of the germ cell population impacts the ability of the testis to produce normal levels of mature sperm, potentially leading to the dysgenesis syndrome correlated with infertility.

The aim of this dissertation is to evaluate the spermatocyte proteins thought to be directly involved in apoptosis following MEHP administration. The proposed mechanism to regulate germ cell death following MEHP exposure describes three important steps in the process: 1) the role of the transcription factor p53, 2) the expression of death receptors in targeted cells, and 3) the modulation of anti-apoptotic protein c-FLIP expressed in germ cells. In addition, the dissertation addresses the cell specificity of testicular apoptosis and novel observations regarding the use of chemotherapeutic treatments in normal physiological tissues.

Chapter 2:
Materials and Methods

I. Materials

2.1 Animals

Approximately 28 day-old C57BL/6J mice were purchased from Jackson laboratories (Bar Harbor, ME). Adult male Fisher rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). Breeding pairs of TRAIL gene deficient C57BL/6 mice (TRAIL-/-) were provided by Amgen Inc. (Thousand Oaks, CA). Breeding pairs of DR5 gene-deficient C57BL/6 mice were provided by Dr. Wafik El-Diery (University of Pennsylvania, Philadelphia, PA). *Itchy* mice were a gift from the laboratory of Dr. Lydia Matestic (University of South Carolina, Charleston, SC) and Dr. Nancy Jenkins (National Institute of Health, Washington, DC) *Itchy* mice are not gene deficient but possess a radiation-induced pericentric inversion on chromosome 2, resulting in the loss of Itch gene functionality (Perry, 1998). Thus, *Itchy* mice are referred to as Itch protein deficient. p53-/- mice were provided by Dr. Lawrence Donehower (UT-MD Anderson, Houston, TX). The mice are described as chimeric: 75% C57BL/6, 25% 129S/v (Donehower, 1992).

All animals were allowed to acclimate for 1 week upon arrival at the University of Texas Animal Resource Center before experiments were performed. The mice were housed under a 12 h light cycle, constant temperature (74±2 °F) and at 30-70% relative humidity. The mice were given water and lab chow ad libitum. All procedures involving animal use were performed in accordance with the guidelines of the University of Texas at Austin's Institutional Animal Care and Use Committee and the National Institutes of Health.

Note: There is a slight difference in the apoptotic response to MEHP in C57/BL6/J mice versus C57/129S/v strains, which are the background of the p53 deficient mice. In the former, apoptosis and caspase-8 activation occurred most

prominently at 12 hours, with a slight trimming effect by 24 h, although cell death is still quite prevalent. The 129S/v mice exhibit a more linear death, also increasing significantly at 12 h but increasing through 24 h. The differences in sensitivity is not known at this time, although the extrinsic pathway is engaged in both strains and the mice express all other phenotypes associated with the MEHP treatments.

2.2 Cell Culture Lines

The GC-1spg (CRL-2053) and GC-2spd (ts) (CRL-2096) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The GC1 cells used are of a spermatogonia lineage and were cultured at 37°C for at least 48 hours prior to treatment exposures. GC-2spd(ts) (a temperature sensitive cell that allows for p53 nuclear localization at 32°C but not 37°C), undergo cell death by addition of various apoptotic stimuli. The cell line also expresses all apoptotic proteins examined in the present study. The GC-2 cell line was originally developed by Hofmann *et al.* as a means to study the process of germ cell differentiation (Hofmann, 1992). The cell line was generated by transforming the SV40 virus' large T antigen into preleptotene spermatocytes of six week old BALB/c mice and subsequently co-transfecting them with a plasmid carrying a *p53* mutant gene ($p53^{Val\text{-}135}$). The mutant p53 protein is fully active at 32ºC, only partially active at 37ºC, and completely inactive at 39ºC. The activity of the protein is determinant on the correct folding of p53, which is inhibited at the higher temperatures. Therefore, the GC-2 line affords experimentally a functional *p53*+/+ or *p53* -/- germ cell model. Furthermore, this cell line is an appropriate *in vitro* model for our purposes since these cells were derived from spermatocytes, the germ cell type most sensitive to apoptosis after toxicant-induced Sertoli cell injury.

II. In Vivo Methods:

2.3 MEHP administration and challenge

All MEHP treatments were administered in mice of approximately 28 days of age. Mice were given a single dose of MEHP (1 g/kg) by oral gavage, a standard dosing procedure for the investigation of MEHP-induced testicular injury (Giammona, 2002). Mice received MEHP in corn oil at a volume of 4 ml/kg. Control mice received a similar volume of corn oil vehicle. Vehicle- and MEHP-exposed mice were killed by CO_2 inhalation at the time points indicated. Both testes were rapidly removed and either rapidly frozen in liquid nitrogen and stored at -80°C or was immersion-fixed overnight in Bouin's solution (Polysciences, Inc., Warrington, PA, washed in 70% ethyl alcohol-Li_2CO_3 (saturated solution; Mallinkrodt, Paris, KY) and embedded in paraffin.

2.4 Primary rat seminiferous tubule explants

Adult male Fisher rats were killed by CO_2 inhalation and their testes were rapidly excised, the tunica albuginea removed, and the remaining seminiferous tubules placed in a petri dish of PBS at room temperature as previously described (Kotaga, 2004). Using the transillumination technique under a dissecting microscope, individual stage VIII-XII seminiferous tubules (representing the tubules thought to be most sensitive to MEHP) 1 mm in length were dissected from the surrounding interstitial tissue with fine stainless-steel forceps and placed in media. Stage VIII-XII segments of seminiferous tubules were chosen for the experiments since these stages contain spermatocyte populations known to undergo death receptor-mediated apoptosis and also because they can be easily and repeatedly identified *via* the transillumination technique (Kangasniemi, 1990). Tubules were added to Sertoli cell media (F-12/DMEM, 1:1) in 6-well

plates and incubated overnight at 32°C. Afterward, 0.5 µg/ml of hTRAIL, and/or 100 µM of the caspase-3 inhibitor z-DEVD-FMK (EMD Biosciences, San Diego, CA) or TRAIL/DEVD was then added to the culture and incubated for 10 h before counting the number of apoptotic cells per tubule. Seminiferous tubules segments were then squash mounted on slides and apoptotic germ cells were labeled by the TUNEL method using the ApopTag kit (Intergen, Purchase, NY).

2.5 *In Situ* Detection of mRNA in Mouse Testicular Tissues

Paraffin tissue cross sections (5 µm) were prepared from testis tissues of wild type mice treated with MEHP as described above. The tissues were then deparaffinized with Citrisolv for 15 m and subjected to a serial alcohol wash (100%, 90%, 70% ethanol). Single stranded oligonucleotide primers (synthesized by IDT Technology, Coralville, IA) were designed to interact with specific DR5 RNA sequences. Primer sequences were: *sense* (5-cgt ctc tct tgg acc gac aga cat cta gca cga caa atg act cta-3) and *antisense* (3-gca gag aga acc tgg ctg tct gta gat cgt gct gtt tac tga gat-5). The primer set was modified with biotin addition to the five prime end, then applied to the tissue in a 200 pmol/mL concentration for 2 h and incubated at 37°C. The tissues were de-proteinized by treatment with protease K (20 µg/ml) prior to primer hybridization. A streptavidin-alkaline phosphotase conjugate was then added to the tissue for a 40 minute incubation at 37°C. Samples were washed twice in a detergent buffer followed by a 20 minute incubation in BCIP/NBT, a colorimetric substrate visualized using light microscopy. All reagents from Maxim Biosystems High Sensitivity In Situ Kit (IHD-0050)

2.6 Immunohistochemical detection of proteins

Wild type male C57BL/6J mice were treated with MEHP or corn oil vehicle as described above. After 24 hours, testis were removed, fixed in Bouin's

reagent and embedded in paraffin before being sliced at 5 µm thickness. Following de-parafinization and hydration, the samples were processed for antigen unmasking using 10 µM sodium citrate and 0.2% Triton X. After blocking with 10% goat serum at room temperature for 20 minutes, the p53 protein was labeled by incubating tissues overnight at 4°C with a goat polyclonal primary antibody (SantaCruz, SC-1312). The primary antibody binding was detected using a donkey anti-goat secondary antibody conjugated with biotin. A horseradish peroxidase kit (Vector Labs, # PK-6102) was used to conjugate biotin with DAB for chemiluminescent detection.

2.7 Morphological evaluation of testicular tissue

Sections (5 µm) of paraffin embedded tissues were evaluated for morphological changes *via* standard methods using periodic acid-Schiffs-Hematoxylin (PAS/H). Tissues were imaged using a Nikon E800 microscope equipped with a digital camera and captured using MetaMorph software (Downingtown, PA).

2.8 Immunofluoresent detection of testicular antigens

Sections (5 µm) of paraffin embedded tissues were also exposed to fluorescent antibody treatment. Following removal of paraffin with citrosolv (15 min), the tissues are heated for 20 min to "unmask" the protein antigens of interest. The tissues were then subjected to .3% triton X before three washes in PBS. Goat serum was diluted 1:10 in PBS and used to block the tissue sections for thirty minutes. A rabbit polyclonal anti-Itch antibody (Abgent, # AP2127a) is applied and allowed to react overnight at 4° C. After three washes with PBS, Alexafluor 488 (1:10,000) is applied for thirty minutes. The slides were then evaluated using the Metamorph software on the Nikon E800 microscope.

III. *In Vitro* Methods

2.9 *In Vitro* culture treatments

GC-1 or GC-2 cells that had been in culture for 48 h (grown at either temperature) were exposed to various treatments including anti-Fas agonistic antibody Jo2 (E-Biosciences), recombinant soluble mouse TRAIL (mTRAIL, cat. #SE-722, Biomol, Plymouth Meeting, PA), recombinant human TRAIL (hTRAIL, provided as a kind gift of Dr. SB Bratton, The University of Texas at Austin), functional grade purified anti-mouse DR5 antibody (Clone MD5-1, cat. #16-5883, eBioscience, San Diego, CA), z-VAD-fmk-FMK (cat. #27610, Calbiochem, La Jolla, CA), cyclohexamide (cat #GR-310, Biomol, Plymouth Meeting PA). Pretreatment of cells with pifihthrine-∝ (AG Scientific, P1177), cyclohexamide (1 µg/ml), pan-caspase inhibitor z-VAD-fmk-FMK (10 µM), or with anti-DR5 antibody (0.5 µg/ml) were added 30 minutes prior to TRAIL addition. Evaluation of cell morphology was performed using a Nikon TMS-100 phase contrast microscope and images were captured using a Nikon CoolSnap digital camera and images were processed using MetaMorph imaging software.

2.10 Immunoprecipitation

GC-2-spg(ts) cells were cultured in 6-well plates (1 X 10^6 cells/well plated) as previously described. Briefly, the cells were cultured for 24 hours at 37° C post split and cultured for an additional 24 hours at 32° C, thus activating the p53 gene. At 48 hours, select wells were treated with 5 µg/mL Fas-activating antibody (JO2) for a period of 3 and 6 hours. For collection, cells were lysed and removed from the wells using RIPA lysis buffer made in the lab (1% IPEGAL, 150 mM NaCl, .5 % sodium deoxycholate, .1% SDS, 50 mM Tris, 8.0 pH) with 1 mM PMSF protease inhibitor added. Lysates were precleared with 50 µl (50 %

slurry) of Protein G sepharose (Amersham) in 500 µl lysis buffer for 1 hour. The supernatent was retained following clearing, and 2.5 ug of rat monoclonal anti-cFLIP antibody was added to the supernatent for one hour at 4 C. A supplementary 50 µl of sepharose was added to the supernatent and incubated for two additional hours at 4º C. The captured antibody-protein G complexes were then washed three times with lysis buffer and once with PBS. After the addition of SDS running buffer, Protein G was removed from the protein by boiling for 5 minutes, at which point the complexes were loaded into a 12% polyacrylamide gel and separated by electrophoresis. The protein was transferred to a nitrocellulose membrane and subsequently probed for with the C-Flip (Dave-2) rat monoclonal (AbCam, ab16078) antibody and Ubiquitin rabbit polyclonal (Stressgen, SPA-200) antibodies.

2.11 Flow cytometric analysis of apoptosis

The annexin-V / propidium Iodide (PI) assay was used to identify apoptotic cells *via* annexin-V's ability to bind to externalized phosphotidylserine molecules (van Engeland, 1998). PI is expelled from live and early-apoptotic cells that maintain their cell membrane integrity, while being retained by dead cells or cells undergoing secondary necrosis/late apoptosis. Cell death is qualified based on their "apoptotic" characteristics (Annexin-V positive stain) using a flow cytometer. After the indicated treatment(s), cells were collected by brief trypsinization (using a 0.25% solution of trypsin with 0.03% EDTA) followed by centrifugation at 400 x g for 6 min. They were re-suspended in fresh media for about 30 min to neutralize the effects of trypsin. The cells were then pelleted by centrifugation at 400 x g and the cell pellets were washed and resuspended in binding buffer as per the manufacturer's instructions (Annexin-V FITC apoptosis kit, cat. #556570, BD Biosciences Pharmingen, San Diego, CA). Approximately 1 X 10^5-10^6 cells/ sample were incubated with 5 µL of annexin-V conjugated with fluorescein isothiocyanate (FITC) for 5-10 min. The cells were

then incubated with 10 µL of PI for 2-5 min. Flow cytometric analysis of these cells was carried out on a Coulter Epics XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

2.12 Flow cytometric analysis of DR5 receptor expression on GC-2 membranes

The DR5-labeling procedures were performed according to the method of Jin *et al.* with modifications (Jin, 2004). GC-1 and GC-2 cells (1 x 10^6 cells) were grown in 6 well plates at either 37 or 32°C. At designated time points, the cells were rinsed with phosphate buffered saline (PBS) and subsequently detached from culture plates using 0.05% trypsin solution containing EDTA. Cells were re-suspended in DMEM and were centrifuged at 4 ºC at 400 x g for 5 min followed by a wash with PBS supplemented with 2 % fetal bovine serum (FBS). The cell pellets were then re-suspended in 50 µl of PBS (with 2 % FBS) containing 0.02 µg/µl of the primary antibody against DR5 (Cat. #210-743, Alexis, CA) or the isotype control (hamster IgG, Pharmingen). Cells were incubated with the primary antibody on ice for 1 h followed by a wash with 1 ml 2% FBS in PBS. The cells were then incubated with a secondary antibody (goat anti-hamster IgG) conjugated to Alexa fluor® 488 (A-21110, Molecular Probes, Eugene, OR, USA) at 20 µg/ml for 1 h on ice in the dark. After washing the cells with 2% FBS in PBS, cells were re-suspended in PBS. Flow cytometric analysis of these cells was then carried out on the Coulter Epics XL-MCL flow cytometer.

2.13 Western blot analysis

All proteins of interest were evaluated in GC-2 cell lines for total cellular protein levels *via* western blot analysis. Western blots were performed using the primary antibodies against the relevant protein in the appropriate dilution

(usually .5 ug/ml). Horseradish peroxidase-conjugated secondary antibodies (SantaCruz Biotechnology) were used to detect primary antibodies. Signals were detected by the use of an ECL kit (Amersham Pharmacia Biosciences, Piscataway, NJ). Images were collected by a Kodak DC-290 digital camera and densitometric analysis was performed using Kodak 1D Image Analysis Software (Kodak Digital Sciences, Rochester, NY). Each protein was analyzed in a minimum of three GC-2 sample sets, and representative blots have been presented. Equal loading (20 µg) was verified by comparing expression levels of b-actin.

2.14 Confocal detection of DR5 on the cell membrane

GC-2 cells were grown at 37°C, trypsinized, and inoculated into four chambered slide plates (Lab Tek II, 125652) at approximately $1.0X10^5$ cells per well. A portion of the cells were grown at 24 h at 37°C, and then transferred to 32°C for 24 h to activate p53. P53 negative cells were kept at 37°C for 48 hours. Both sets of cells were then treated with 0.5 µg/ml of MD5-1 for 30 mins, followed by treatment with 0.5 µg/ml of mouse TRAIL. Both sets of cells were collected at 2 h time points. Cells were then fixed in ice-cold 1:1 methanol/acetone for 10 minutes and air-dried. Following three washes with PBS and a 20 min blocking step in a 10% goat serum, the cells were incubated overnight in DR5 (Abcam, # ab8416) primary antibody diluted to a 1:100 ratio. The cells were then washed thrice with PBS and goat anti-rabbit Alexa Fluor 488 (Alexis) secondary antibody was added in a 1:1000 ratio. The slides were then mounted using VectorShield mount (VectorLabs, # H-1300) containing PI to stain the nucleus. Fluorescing cells were evaluated using the Leica SP2 AOBS confocal microscope and images were taken at 40X and 63X.

2.15 Use of siRNA to knockdown Itch protein

In 12-well plates, approximately 7 x 10^4 GC-2 cells were plated per each well. Mixed with the cells was a cocktail of serum-free OPTI-MEM (Gibco), 30 nM of Itch siRNA, and the Amine transfection agent. The cocktail was activated by initially combining the transfection agent and OPTI-MEM at room temperature for 10 minutes. This mixture was then combined with appropriately diluted siRNA (30 nM final concentration) for an additional 10 minutes at room temperature. Gently the cocktail and GC-2 cells were combined and allowed incubate for 24 hrs at 37° C. One plate was then placed at 32° C for an additional 24 hrs. Media was replaced at the 24 hour time point and protein knockdown was evaluated by western blotting 48 hours after initial transfection. The Itch siRNA was designed and synthesized by Ambion (Austin, TX) and the Easy Silencing kit (Ambion # 1640) was used to complete the transfection.

2.16 Protein detection of c-FLIP using silver staining

Following immunoprecipitation (described above) of FLIP, the pull-down products were loaded into a 10% Bis-Tris acrylamide gel and run for 1 hr at 130 milliamps. The gel was then transfered from the electrophoresis apparatus into a clean plastic dish for 20 m incubation in a fixing buffer (40% methanol, 5% acetic acid, 55% water) at a gentle rotation. Sensitizing solution is added between two washes of ethanol (30%) for 10 m a apiece, then water for 10 m. A silver ion staining solution is then added for 15 m, followed by a brief rinse, and the introduction of developing solution for approximately 10 m. Once the silver stain is clearly observed, a stop buffer is added to prevent overexposure. All buffers and stains (except for fixative and ethanol washes) were found in the Silverquest Silver Stain kit (Invitrogen, # LC-6070). Photos were taken using the Kodak 1D Image Analysis Software (Kodak Digital Sciences, Rochester, NY).

2.17 Statistical methods

Significance between groups ($p < 0.05$) was determined by using the parametric single factor analysis of variance (ANOVA) test, followed with the Fisher PLDS post-hoc comparison test using Statview software (SAS Institute Inc, Cary, NC).

Chapter 3:
p53 activity plays a role in the apoptosis of germ cells

3.1 Introduction and Rationale

The involvement of the p53 protein in the apoptotic pathway is well documented. This transcription factor has been implicated in cell cycle arrest, apoptosis following DNA damaging agents such as ionizing radiation and reactive oxygen species, as well as in a variety of non-genotoxic stressors including hypoxia (reviewed in Lavin and Guerin, 2006). In addition, p53 can mediate cell death by regulating proteins of both the intrinsic and extrinsic apoptotic pathways.

The activity of p53 in germ cells is known to be indispensable in the proper regulation of cell cycle progression during mitosis and meiosis, two processes key to spermatogenesis, and appears to be especially important in spermatocytes (Almon, 1993), (Rotter, 1993). p53 deficient mice are fertile but exhibit defects in spermatogenesis, including increased levels of germ cell degeneration (Rotter, 1993). However, the function of p53 in toxicant induced apoptosis is unknown. Testicular apoptosis can be triggered under a variety of conditions, resulting in changes to germ cell or Sertoli cell homeostasis. Heat, radiation, and torsion are examples of stress models that directly effect the germ cell, promoting death by damaging DNA or reducing oxygen to the tissues (Embree-Ku, 2002), (Lysiak, 2000).

MEHP, however, is known to specifically target and damage Sertoli cells. Because of its established role in providing support for up to thirty germ cells at a given time, any change in Sertoli cell homeostasis potentially impacts germ cell numbers as a secondary event. Increased membrane expression of death ligands TRAIL and FasL is one possible consequence of Sertoli cell damage (Boekelheide, 1998). Withdrawal of growth factors or nutrients from the germ

cell may also facilitate the early stages of its apoptosis. An important question not currently understood is how the germ cell senses and responds to these stresses.

The p53 protein has been implicated as a factor that responds to testicular stress (Yin, 1997). The protein exerts a measure of regulatory control over the expression of intrinsic pro-apoptotic proteins Bax, Noxa, and Puma. However, there is limited data reporting initiation of mitochondrial apoptosis in germ cells other than after DNA damage (Erster, 2004). Genotoxic stress may also be transcription-independent, with p53 localizing to the mitochondria in a mechanism that blocks bcl-2 and allows for the release of cytochrome c (Erster, 2004). On the other hand, p53 transcriptional targets associated with the extrinsic pathway may be increased after toxicant-induced stress in germ cells (Chandrasekaran, 2005), (Giammona, 2002). An indirect link to p53 transcriptional activity was previously established when Lee *et al.* determined that the Sertoli cell toxicant MEHP increases Fas expression in the testis (Lee, 1997). Management of many p53-dependent apoptotic proteins is thought to be achieved largely at the mRNA level. The genes for Fas and DR5 both contain a p53 response element, thus placing them under the transcriptional control of the protein (Owen-Schaub, 1995), (Wu, 1997). Furthermore, the functional activity of p53 is correlated with the expression of death receptors in well established models as heat stress and ionizing radiation in the testis (Boekelheide, 2005), (Miura, 2002).

Controlled expression of pro-apoptotic proteins preserves a balance between death and survival. New evidence suggests that cells commonly removed in the “first wave” of apoptosis in developing testis may be driven by Fas and other proteins within the extrinsic pathway (Lizama, 2006). Thus, death receptor expression could be essential for the routine development of normal spermatogenesis. In contrast, its misregulation is commonly associated with testicular disease. For instance, cryptorchidism, a developmental defect of the testis and a component of testicular digenesis syndrome, is strongly correlated with p53 and Fas over-expression (Yin, 2002).

Cellular stressors in the testis may lead to the loss of germ cells and impediment of spermatogenesis. The focus of this chapter evaluates the possibility that MEHP mediated Sertoli cell damage is influenced by p53 present in germ cells, leading to its increased activity and transactivation of pro-apoptotic proteins, specifically in spermatocytes. The spermatocyte subtype represents the main population of germ cells residing in the adluminal space and as a result is the most likely cell type to be effected by MEHP treatments. The p53 protein has been suggested as a "guardian" of germ cell integrity, potentially reducing damaged or degenerate cells that may inhibit spermatogenesis (Yin, 2002). The hypothesis here assesses whether p53 transcriptional activity is an important physiological response in germ cells that have been treated with MEHP and if its a major determinant of their sensitivity to apoptosis.

Although MEHP is an established Sertoli cell damaging agent, its exact mechanism of inducing germ cell apoptosis is speculative. Here we present evidence that the p53 protein is stabilized in testicular germ cells following MEHP treatments and that this stabilization results in the increased production of death receptors Fas and DR5. This association between p53 and death receptor expression can be observed both *in vivo* and *in vitro* and correlates with increased incidence of apoptosis, specifically in the spermatocyte. In p53-/- mice or cultured germ cells grown at p53 non-permissive temperatures, the induction of death receptors is reduced and overall less caspase-8 activation is seen. The data indicates that p53 response is an important factor in producing an extrinsic apoptotic response in germ cells following MEHP toxicant challenge.

3.2 Results

A. MEHP induces apoptosis in the seminiferous tubules of C57/B6/J mice

Exposure of C57/BL6/J mice to a standard MEHP treatment by route of oral gavage induces cell death in the seminiferous tubule (**Fig 3.1**). A comparison between control and MEHP treated mice shows that the tubules of the latter reveal higher levels of germ cell apoptosis, retraction of the Sertoli cell cytoplasm, increased vacuolization, larger lumens, and fewer overall germ cell numbers (see **Fig. 3.1** legend). This data is consistent with earlier findings that MEHP treatments can lead to Sertoli cell damage and potential for impaired spermatogenesis, testicular dysgensis syndrome, or both. *In situ* DAB staining (TUNEL) shows that cell death increases in the seminiferous tubules when treated with MEHP and that the apoptosis was concentrated chiefly in pachytene spermatocyte and possibly early round spermatid populations.

B. p53 protein levels are stabilized or increased in spermatocytes after MEHP exposure

Evaluation of p53 protein expression by immunohistochemistry in testicular tissue sections reveals a low level of p53 distributed throughout the spermatocyte population of the seminiferous tubules of non-treated animals (**Fig. 3.2A**) In the testis from MEHP-treated mice, p53 levels were found to be considerably elevated specifically in spermatocyte populations by 24 h (**Fig. 3.2A**). Spermatocytes are among the most sensitive populations of germ cells to undergo MEHP-mediated apoptosis, as observed using TUNEL *in situ* hybridization (**Fig. 3.1**). Morphological changes are consistent with the retraction of the Sertoli cell membrane following MEHP treatment and p53 protein is localized to a ring of spermatocytes along the basement membrane. Thus, p53 expression and apoptosis appear to be correlated in spermatocytes.

To determine if p53 is stabilized through post-translational modification, MEHP-treated testicular samples were immunoblotted and probed for changes in phosphoylation status (**Fig 3.2B**). Phospho-serine-18 (mouse specific), known to disrupt the association of p53 and its inhibitor, MDM-2, were not observed with or without MEHP. Phospho-serine-18 was detected in UV-treated (30 m, 254 nm) GC2 cells used as a control. However, phosphorylation of p53-serine-392, known to increase p53 nuclear localization and apoptosis, was detected and increased following MEHP in wild type mice.

C. Increases in p53 transcriptional activity correlate with higher death receptor expression *in vivo* and *in vitro*

Western blot analysis of wild type testis showed an increase in Fas death receptor expression concomitant with longer exposure to MEHP. Fas levels in p53 deficient mice did not significantly increase, although initial concentrations are detectible in the tissue lysate (**Fig. 3.3 A**). GC-2 cells showed differences in the amount of the death receptors expressed between cells that express large amounts of functional p53 (32° C) or low amounts (37°C) (**Fig. 3.3B**, for both 32° & 37°C). Both Fas and DR5 total cell protein is elevated at the permissive temperature (32° C)

Immunoblotting also showed the abundant expression of the p53 dependent p21 protein only in GC-2 cells maintained at the permissive temperature, indicating the ability of p53 to enter the nucleus and up-regulate its target genes (**Fig. 3.3B**, p21 row). Previous observations showed the phosphorylation of p53 on serine-392 is associated with its stabilization in the nucleus and was shown to increase after MEHP exposure *in vivo* (**Fig. 3.2B**). The data indicated that p53 nuclear translocation is potentially important for its cytotoxicity. In keeping with this hypothesis, the expression of p53 sensitized GC2 cells to apoptosis. Cells grown at the p53 permissive temperature underwent greater apoptosis (19.5%) than those at the non-permissive (5.2%) as determined

by annexin-PI flow cytometry (**Fig. 3.4A**). Addition of a Fas cross-linking antibody (Jo2) induced further death at both temperatures, but more meaningfully when p53 is expressed at a higher rate (32.3%).

The p53 blocking compound Pfithrin-∝ (PFT-∝, 50 μgs) was used to decrease the ability of p53 to enter the nucleus and test if GC2 cells were protected from cell death. Western blotting showed enhanced Fas expression at the p53 permissive temperature with no treatment. However, pre-treatments with PFT-∝ reduced Fas protein almost to the level detected in p53 non-permissive cells, consistent with a reduction in p53-mediated transcription (**Fig. 3.4B**). The results of annexin-PI flow cytometry shows that in PFT-∝ pre-treated, samples show decreases in Fas that may explain protection of GC2 cells from apoptosis at the p53 permissive temperature (**Fig. 3.4C**). This data suggests a correlation between p53, the increased expression of death receptors, and apoptosis in spermatocyte germ cells.

D. <u>MEHP promotes apoptosis through the extrinsic pathway and is p53 dependent</u>

Treatment of wild type or p53 deficient (-/-) mice with MEHP activates caspase-8, an integral protein in the extrinsic pathway. After treatment of both wild type and p53-/- mice with 6, 12, or 24 h with MEHP, changes in protein levels were determined by western blotting. Processing of caspase-8 was shown to increase as early as 12 h and continue through 24 h. (**Fig. 3.5A**) The active 18 kD sub-unit was detected in the lysates of all wild type animals, but was significantly increased following MEHP in the 12 and 24 h time point. A lack of activation was shown in the cells of p53-/- mice, indicating that without the transcription factor, very little caspase-8 is activated following MEHP. The full length procaspase-8 band (56 kD) the major product in these mice, although the primary processing band(s) (p43/41) and the activated p18 bands were also faintly visible.

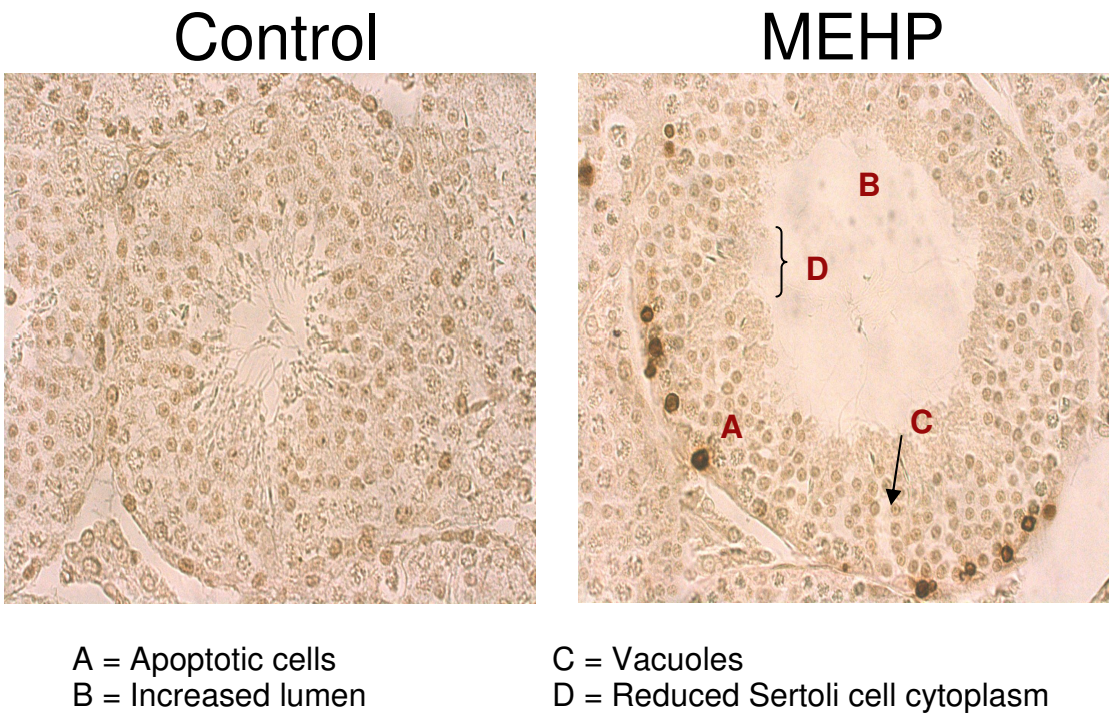

Fig.3.1 *MEHP Induces Testicular Apoptosis in C57/B6/J Mice.* Exposures of MEHP by oral gavage induces apoptosis in the testis of wild type mice. MEHP causes various morphological disturbances to the seminiferous tubules, including reduced Sertoli cell cytoplasm, increased lumens, and vacuolization in addition to increased incidence of apoptosis. Positive TUNEL staining (brown) is specific to spermatocyte populations.

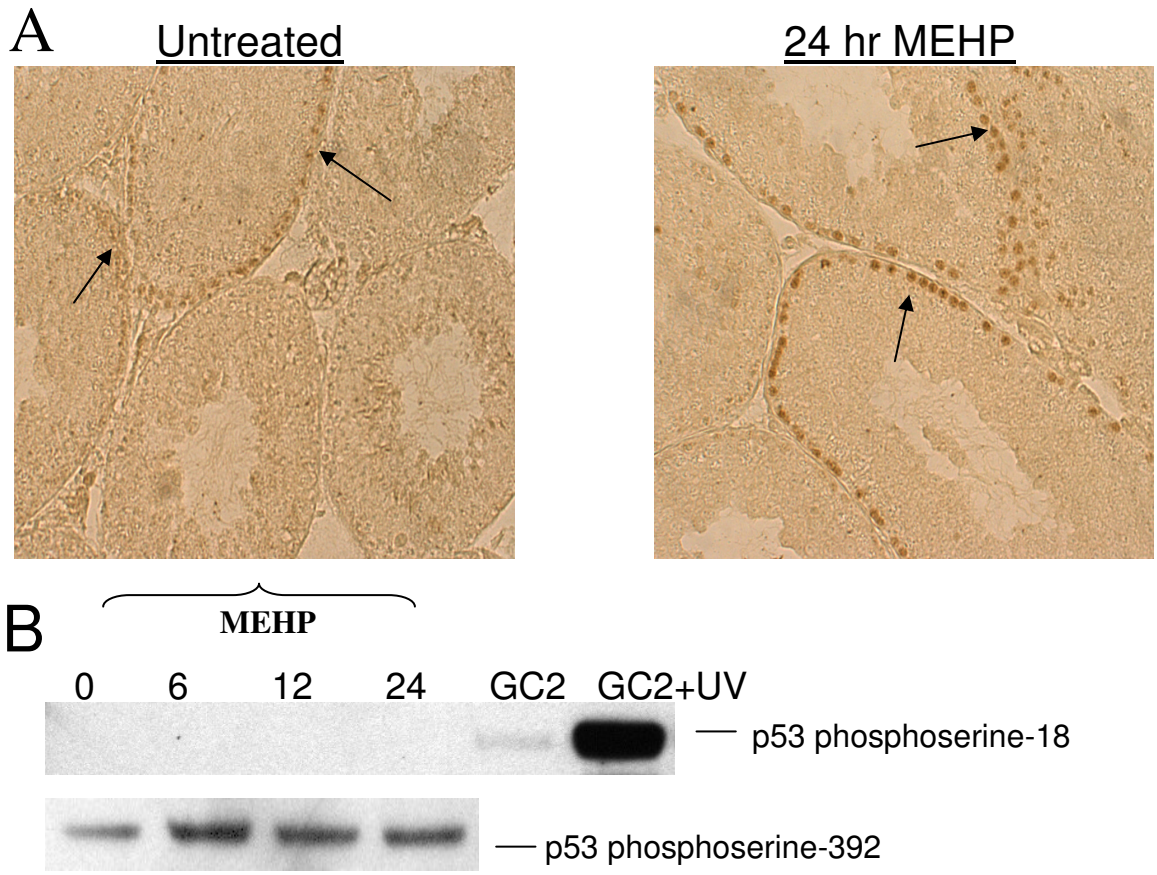

Fig.3.2 *MEHP Stabilizes p53 Expression in Spermatocytes of C57/B6/J Mice.* (**A**) Immunohistochemistry of mouse testicular tissue following a MEHP treatment. Control mice show general p53 staining distributed among spermatocyte populations in the tubule. Animals treated with MEHP for 24 h show robust p53 staining (arrows) specifically in spermatocytes. (**B**) p53 is phosphorylated on serine-18 (mouse specific) only in UV-treated GC2 cells and not *in vivo*. Serine-392 is expressed constitutively and increases over a 24 h time course treatment with MEHP.

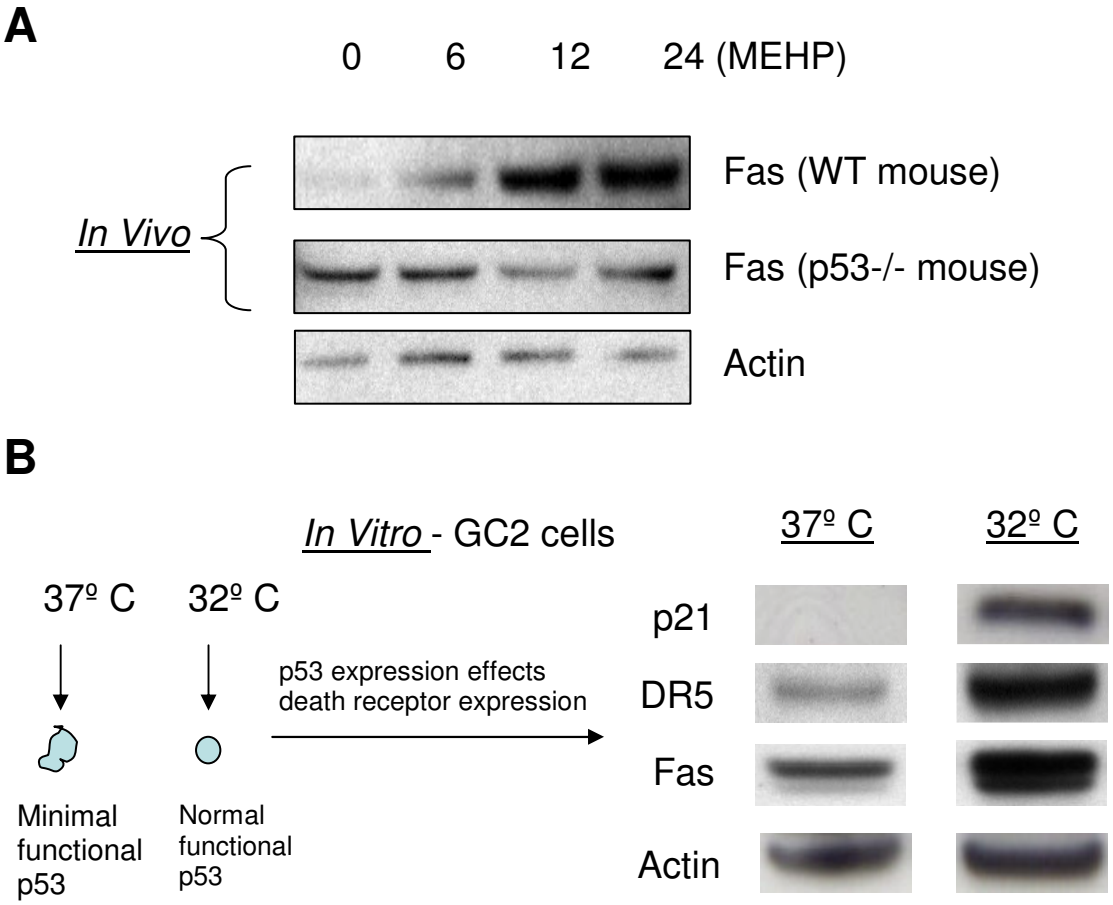

Fig. 3.3 *Expression of death receptors is p53 dependent in vivo and in vitro.* **(A)** Wild type mice treated with MEHP show increases in Fas death receptors, but p53-/- mice are protected. **(B)** Spermatocyte-like GC2 cells also express death receptors Fas and DR5 in a p53 dependent manner. The expression of increased p21 also indicates p53 translocation into the nucleus.

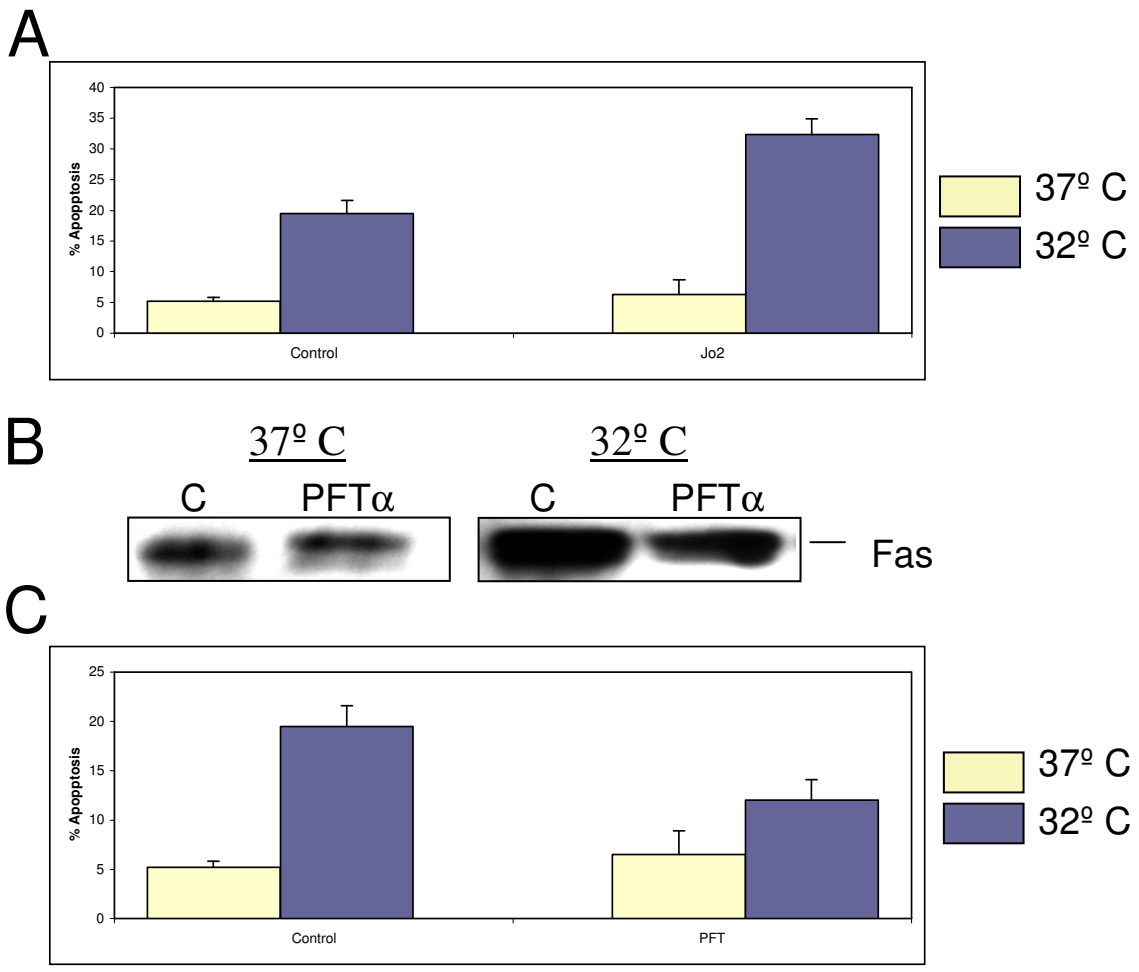

Fig. 3.4 *p53 activity correlates with Fas expression and increased apoptosis in GC2 cells*. **(A)** Cell death is higher in GC2 cells grown at p53 permissive tissues than at non-permissive temperatures and is increased by Fas-linker Jo2. **(B)** Pifithrine-α pre-treatments reduces Fas expression in GC2 cells when p53 levels are high, suggesting transcriptional regulation. **(C)** Reduced Fas expression corresponds with less apoptosis as determined by flow cytometry.

E. MEHP treatment increases DR5 mRNA in spermatocytes:

To test the cell specific changes in DR5 mRNA after MEHP, *in situ* hybridization was performed. DR5 mRNA in testis from untreated mice could be detected in spermatocytes (**Fig. 3.6A**). However, DR5 mRNA levels are increased in the testis as early as 3 h (**Fig. 3.6B**) following MEHP treatment and were maintained through the latest time point collected (24 h, panel D). The expression of DR5 in germ cells from treated mice was most prominent in early spermatocytes that became located along the basement membrane of the seminiferous tubule due to the retraction of the Sertoli cell membrane. MEHP is known to induce alterations in Sertoli cell structure in rodents such as retraction of the Sertoli cell plasma membrane, evident here in all treated tubules, especially at 24 h. (**Fig. 3.6D**). PAS/H staining and microscopy identified the stage (maturity) of effected germ cells. High levels acrosomal staining and hemotoxylin detected the condensed chromatin pattern seen in spermatocytes. Further analysis confirmed that the majority of the cells as pachytene spermatocytes most often associated with an early stage of mouse spermatogenesis (**Fig. 3.6, E & F**)

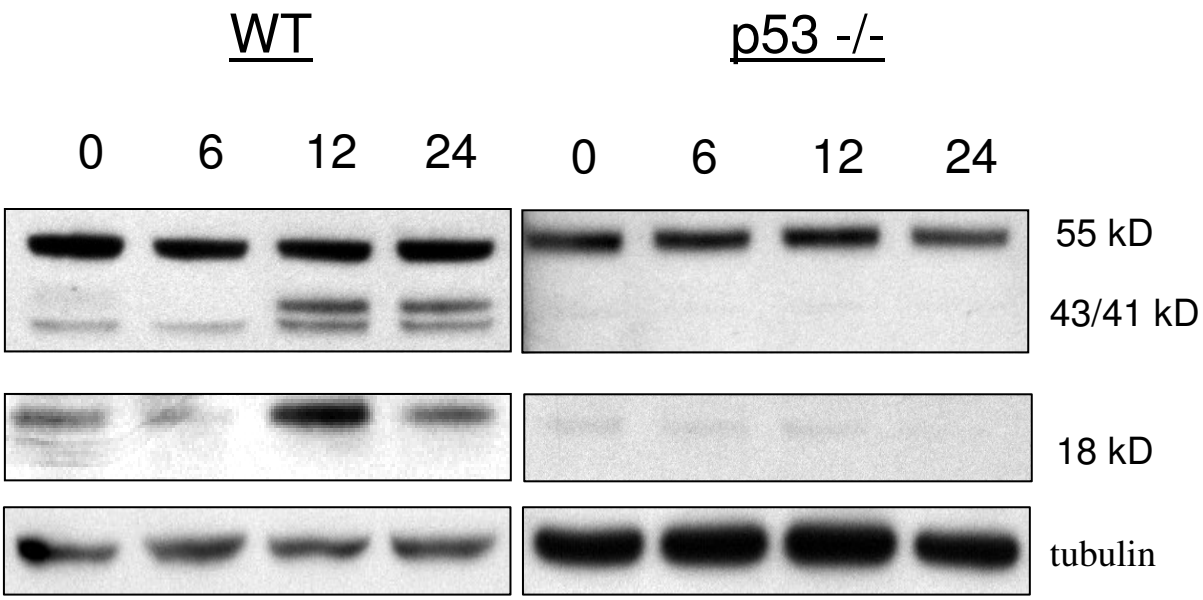

Fig. 3.5 *Caspase-8 is activated in vivo following MEHP exposure*. In wild type mice, caspase-8 processed from (p43) and active fragment (p18) is detected at higher levels following toxicant treatment. Mice deficient in p53 did not express significant amounts of caspase-8 activation after MEHP.

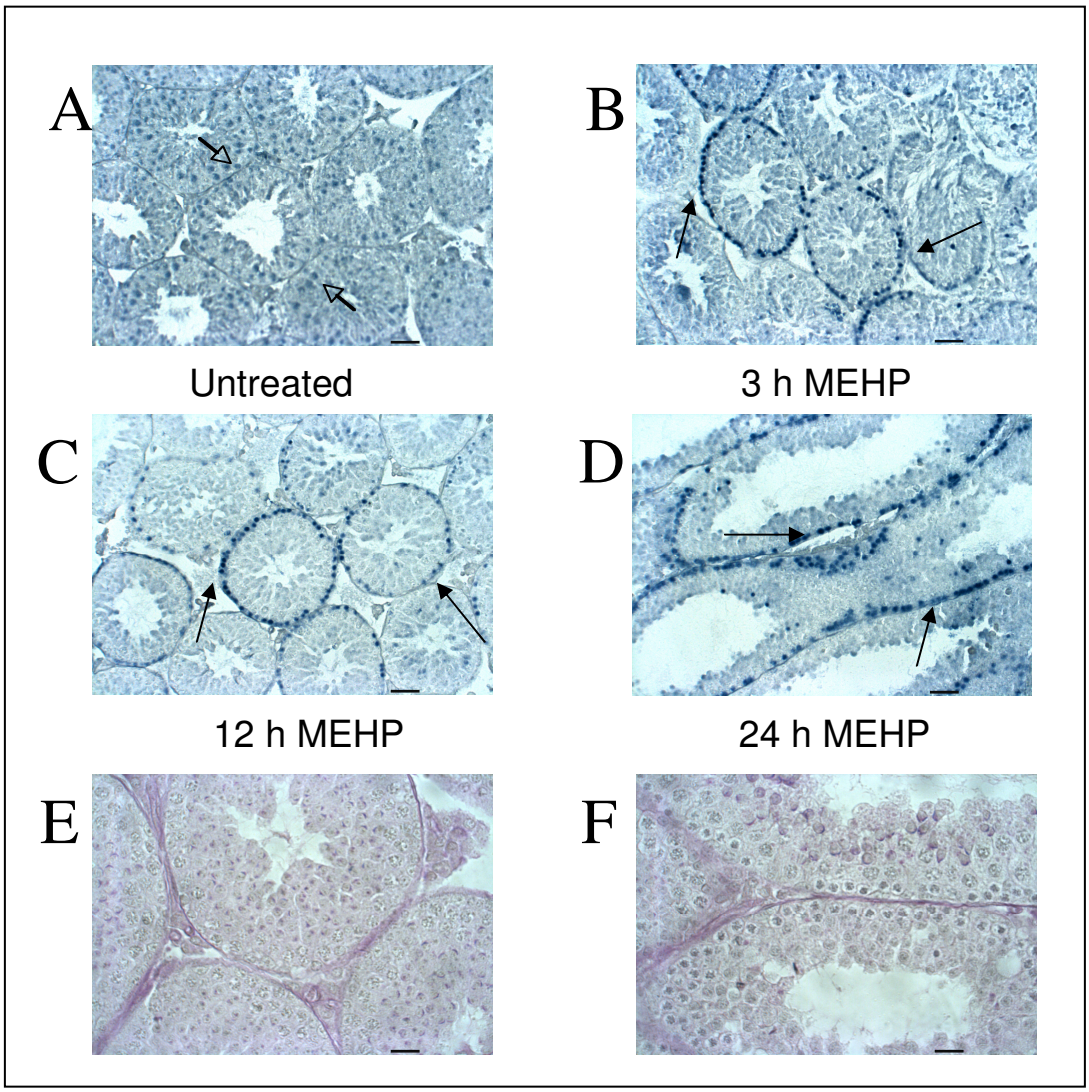

Fig. 3.6 *MEHP treatment of C57/BL/6 mice increases DR5 mRNA expression in germ cells.* Localization of DR5 in germ cell subtypes determined *via in situ* hybridization of testis from vehicle- or MEHP-treated of wild type mice. Representative testis sections from mice exposed to vehicle **(A)**, or MEHP for 3 h **(B)**, 12 h **(C)**, or 24 h **(D)** are shown. Hybridization of primer RNA occurs generally in the spermatocyte population of germ cells in the testis of vehicle-treated mice (A). Significant increases in DR5 expression are seen in testis by 3 h following MEHP treatment in early spermatocytes. Morphological assessment of the tissue was achieved using PAS & H staining (**E** and **F**). Bar equal to 100 µm.

3.3 Discussion

Previous investigations have evaluated the functional role of MEHP in the induction of germ cell apoptosis (Oishi, 1980), (Boekelheide, 1989). There is substantial evidence suggesting that MEHP and other phthalates actively target and promote damage to the Sertoli cell, which directly controls the number germ cells that can be maintained under physiological conditions (Richburg, 1996), (Richburg, 2002). Germ cells not sufficiently supported are thought to be removed from the testis through the process of apoptosis. Thus, upon damage, the Sertoli cell expresses the death ligands FasL and TRAIL, which interact with their cognate receptors found on the apoptotic germ cell membranes (Grataroli, 2002), (Giammona, 2002). Germ cell numbers will decline as a function of Sertoli cell health and ultimately result in decreased incidence of spermatogenesis and increased dysgenesis/infertility. While it has been reported that p53 and death receptors are involved in germ cells targeted for apoptosis after MEHP, the mechanism by which they do so remains unsolved.

Chandrasekaran and Richburg have shown that the sensitivity of germ cells to undergo apoptosis after toxicant-induced testicular injury may be dependent on p53 status (Chandrasekaran, 2005). The linkage between p53 activation in the cell and its sensitivity to death receptor activation was indicated when a lower incidence of basal apoptosis was observed in p53 null (-/-) mice than wild type controls. In addition, mice deficient in the expression of functional p53 showed less incidence of cell death after treatment with the prototypical Sertoli cell toxicant MEHP. The reduced sensitivity to apoptosis was correlative with lower activation of the death receptor DR5, as western blot analysis indicated that the levels of this protein expression in testis cell membrane fractions from MEHP-exposed p53-/- mice were lower compared to those of wild-type mice. Both DR5 and Fas were found to be substantially elevated and localized in germ cell membranes after MEHP treatments. The authors suggested

that p53 may act to facilitate movement of death receptors from the cytosol to the membrane in a transport capability.

Further investigation in this study has shown that MEHP may in fact act to increase or stabilize p53 protein following exposure to the toxicant. Immunohistochemistry of toxicant treated mouse tissue display higher concentrations of p53 in spermatocytes, the germ cell found to be most sensitive to apoptosis (**Fig 3.2A**). Whether the staining is cytoplasmic or nuclear is unknown. However, in mouse testis, the p53 protein is known to reside in close proximity to the nuclear envelope, for rapid transit into the nucleus and subsequent transcription of apoptotic factors upon stress. (Yin, 1997) Morphological analysis of the tissues suggested that the germ cells were pachytene spermatocytes from stages seven or eight. These are the same subtypes most often undergoing apoptosis after MEHP (**Fig 3.1**). Spermatocytes may be particularly sensitive to toxicants due to its location in the adluminal compartment and because it is the site of the synapse and genetic recombination. (Odorisio, 1998)

P53 is known to be stabilized through the post-translational phosphorylation of one or more amino acid (usually serine) sites on the protein. Analysis of different potentially phosphorylated serine residues show that MEHP does not induce modification of serine-18 (mouse specific) (**Fig. 3.2B**). This modification (along with serine-20 in humans) are known to interfere with the binding of MDM2, the primary regulator of cellular p53 levels (Unger, 1999). However, phosphoserine-392 was present constitutively and increased with administration of the toxicant. Modification of this residue is associated with higher p53 stability, and the induction of apoptosis (Sluss, 2004), (Kim, 2004). Phosphorylation of serine-392 possibly aids in the tetramerizaion of p53, thought to be a requirement for proper DNA binding and gene transactivation (Sakaguchi, 1997). Interestingly, phosphorylated serine-392 has been shown to interfere with the ability of p53 to leave the nucleus once modified. The result supports the possibility that p53 protein is imported to the nucleus, where it is stabilized by the

lack of interaction with the MDM2 regulator. In addition, increased DNA binding could lead to transcription of apoptotic gene targets.

The p53 protein is known to promote apoptosis in tumor cells and is thought to at least partially control Fas and DR5 transcription (Wu, 1999), (Munsch, 2000). However, a lack of consensus is displayed in the literature in concern to whether or not p53 is important in driving death receptor system in germ cell apoptosis. The connection between p53 expression and death receptors has been previously explored in our lab using p53 deficient mice (Chandrasekaran, 2005). While MEHP-treated mice show a linear and potent increase in Fas protein as determined in a 24 h time course, Fas expression in p53-/- mice is nearly flat and does not increase significantly (**Fig. 3.3A & B**). The presence of the death receptor in p53 -/- mice shows that it is not totally dependent on p53 transcription. However, p53 must be active for Fas protein levels to increase after MEHP and to induce apoptosis. Therefore, p53 may be important for the regulation of Fas as well as influencing its expression. Bennett *et al.* described a system by which p53 helped traffic death receptors from the golgi for placement on the plasma membrane (Bennett, 1998).

To fully elucidate the pathways involved, we initiated the use of the transformed, spermatocyte-like cell line GC2. GC2 cells are useful for several reasons. Firstly, the cells express all of the proteins associated with extrinsic apoptosis, including death receptors. GC2 cells are derived from a spermatocyte lineage, which is the germ cell sub-type most commonly sensitive to apoptosis post-MEHP exposure. In addition, the cell line contains a temperature sensitive mutation that results in the expression of properly folded p53 protein at the permissive temperature (32° C) and less so at the non-permissive temperature (37° C). By taking advantage of this mutational "toggle," we could create an *in vitro* system that modeled p53+/+ and p53-/-germ cells.

The GC2 cells were first used to establish whether p53 expression was correlative with increased apoptosis. Because MEHP is know to primarily effect the Sertoli cell, and has no effect when directly applied to cultured cells, the

agonistic antibody Jo2 (anti-CD117) was used to mimic the contribution of the death receptor system. Jo2 acts to link Fas receptors together in a manner that simulates *in vivo* associations after triggering by FasL. In doing so, "clusters" of receptor/ligand multimers can be linked together, theoretically increasing the chances of robust apoptosis.

By transferring the GC2 cells from 37° C to 32° C for 24 hours, we sought to potentially model in cell culture the same stimulatory effect of MEHP on p53 protein levels seen in mouse tissues. The increased expression of p53 at the permissive temperature led to a robust and continuous apoptosis compared to that of the non-permissive temperature, supporting previous data obtained in the lab (**Fig. 3.3A**). The experiment also supports the hypothesis that a sharp increase in p53 activity results in germ cell apoptosis. The effect is amplified by the addition of Jo2 and suggests that the death receptors are an integral part in promoting apoptosis in spermatocytes.

To determine if death receptor expression contributed to apoptosis following p53 activation, western blots were performed from GC2 lysates at both temperatures. Analysis of the results showed considerable increases in the protein levels of both DR5 and Fas at the p53 expressing temperature compared to the non-expressing temperature. (**Fig. 3.3B**) As seen *in vivo*, Fas is present at a low basal level, and DR5 shows a similar profile. Both proteins are amplified at the permissive temperature, supporting the proposition that death receptors are activated upon p53 activation in spermatocytes and lead to the extrinsic apoptotic mechanism.

To further investigate the link between p53 and death receptors, GC2 cells were treated with Jo2 anti-Fas agonistic antibody for 6 hours. The cells displayed the most robust cell death coincident with p53 activity. (**Fig. 3.4A**) Pre-treatments of GC2 cells with nuclear pore inhibitor Pifithrin-∝, which blocks p53 entrance into the nucleus, reduced the level of cell death observed at the permissive temperature (**Fig. 3.4B**). The decrease in cell death is coincident with reduction of Fas receptors in the same samples. GC2 cells pretreated with Pifithrin-∝ were

able to decrease Fas levels at the permissive temperature. Thus, the ability of p53 to transactivate Fas is positively associated with germ cell death (**Fig. 3.4C**). The results are interesting in light of the up-regulation of phosphoserine-392 levels observed in mice, which increases p53 nuclear activity (**Fig. 3.2B**).

Activation of caspase-8 increases in response to MEHP treatments *in vivo* (**Fig. 3.5A**). The data correlates with the very high levels of Fas detected at 12 hours post MEHP and continue through 24 hours in wild type mice (**Fig. 3.3A**). The increase in Fas and activated caspase-8 suggests that both of the proteins and the extrinsic apoptotic program are a requirement for efficient apoptotic triggering in germ cells after toxicant treatment.

In summary, p53 appears to be stabilized through phosphorylation in response to MEHP treatments and subsequently exerts an influence over death receptor expression in the testis *in vivo* and *in vitro*. Increased death receptors lead to the cleavage and activation of caspase-8, an initiator of the extrinsic pathway of apoptosis. Low constitutive levels of Fas and DR5 can be detected prior to MEHP. However, with the stabilization of the p53, Fas protein is increased *in vivo*. The death receptors increases seen *in vitro* are probably under the transcriptional influence of p53, which is known to localize to the nucleus in GC2 cells at the permissive temperature (Chandrasekaran, 2005). DR5 mRNA is also up-regulated by MEHP and expressed in spermatocytes.

Previously the Richburg lab examined the protection of *gld* and *lpr* mice against apoptosis triggered by MEHP. The mice have mutations in the FasL gene and Fas receptor gene respectively. Removal of either the receptor or ligand provided reduced sensitivity to apoptosis. Nevertheless, total protection to MEHP-mediated apoptosis was not observed, leading to the hypothesis that another apoptotic factor was alternately expressed. Furthermore, the experimental results also showed a statistically relevant increase in DR5 proteins, although the mechanism of the protein's increase was unknown. *In situ* experiments completed here show that mRNA levels increase in mice treated with MEHP (**Fig. 3.6A-D**). Testicular mRNA was elevated by three hours and remained at robust levels

through 24 hours. Western blotting shows that Fas and DR5 also increases over the same general time interval (**Fig. 3.3A**). DR5, like Fas, is known to promote apoptosis through the DISC which can explain the induction of caspase-8 activation.

The question of why both death receptors would be heavily expressed in the same tissue is a source of continued research. In fact, the TRAIL/DR5 system is rarely engaged in normal physiological cells and preferably induces cell death in tumor cells. However, a hint may lie within the particular sensitivity of spermatocytes to apoptosis. These cells represent the meiotic component of spermatogenesis and are known to be susceptible to wide range of toxicants, especially those that induce DNA damage and possible cell cycle arrest. DR5 is known to be under the control of p53, either by direct transcription or an indirect regulation, and DR5 levels are increased in the same spermatocytes that exhibit p53 stabilization. Because increases in DR5 mRNA in response to MEHP is a novel and intriguing observation, we wanted to look more closely at the protein and its expression in the germ cell. In doing so, the importance of the TRAIL/DR5 system in physiological cells may be revealed, as explored in the next chapter.

Chapter 4:
Sensitivity of Spermatocytes to TRAIL/DR5 Mediated Apoptosis

4.1 Introduction and Rationale

The TRAIL/DR5 tandem is a cell death inducing system analogous to FasL/Fas. Both represent a ligand/receptor interaction that triggers activation of the DISC proteins and apoptosis mediated through the caspases of the extrinsic pathway. However, TRAIL/DR5 possesses an intriguing specificity for tumorigenic cells and under most circumstances is inactive in physiologically healthy cells (Meurette, 2006), (Kim, 2000). For this reason, controlled engagement of the system has been proposed for use as a cancer chemotherapeutic strategy (Ichikawa, 2001), (Kelley, 2004), (Guo, 2005). As observed in the previous section, DR5 mRNA and its protein product is expressed at high levels in germ cells following MEHP and is correlative with apoptosis. An understanding of the mechanisms that confer this sensitivity of germ cells to TRAIL-mediated apoptosis, which is usually limited to the killing of tumor cells and not the inappropriate apoptosis of 'normal' cells, would be useful when investigating therapeutic applications. However, the instigation of TRAIL/DR5 may damage tissues not undergoing tumor growth. This may be particularly important for cells with high proliferation rates such as the testis, ovary, skin and bone marrow.

The functional importance of the death receptor signaling system in controlling testicular germ cell apoptosis has been revealed previously using a toxicant model system (Giammona, 2002). Exposure of rodents to mono-(2-ethylhexyl) phthalate (MEHP), leads to a robust increase in germ cell apoptosis in rats and mice (Boekelheide, 2005). Although it is well established that the cellular target of MEHP is the Sertoli cell, the identity of the specific intracellular target has remained elusive and MEHP-induced injury does not lead to the death of the Sertoli cell themselves. Rather, specific populations of germ cells, the

spermatocytes and possibly early round spermatids, undergo apoptosis (Giammona, 2002). Since the functional capacity of the Sertoli cells is critical in orchestrating the complex process of spermatogenesis and maintaining the viability of the germ cells, it is hypothesized by our group and others that Sertoli cells instigate apoptosis as a mechanism to reduce the population of germ cells to match the reduced supportive capacity (Boekelheide, 2000), (Richburg, 2002).

The death receptor DR5 and its ligand TRAIL have been described to be highly expressed in the testis of rodents (Grataroli, 2002). Previous observations have shown that increases in the expression of DR5 and TRAIL occurs in the testis of MEHP-exposed wild-type and *gld* mice, indicating the possibility that TRAIL/DR5 may also play a role in promoting germ cell apoptosis in the testis and providing the rationale for the present investigation (Richburg, 2000). As seen in the previous chapter, MEHP appears to also increase p53 stabilization and higher transcription of DR5 mRNA. This linkage between p53 status and the expression of DR5 levels could be an important determinant of germ cell sub-type specific apoptosis. Accordingly, in the present chapter, both *in vivo* and *in vitro* experimental model systems are utilized to investigate if conditions resulting in the activation and/or stabilization of the p53 protein both influence the expression of DR5 on the germ cell plasma membrane and promote apoptosis in discrete testicular cell populations.

The experiments also sought to determine, for the first time, that germ cells, in particular spermatocytes, are able to undergo TRAIL-mediated apoptosis and whether pretreatment with agonistic DR5 antibodies synergistically increase their sensitivity to TRAIL-mediated apoptosis. Such a finding would have implications for current therapeutic approaches that combine DR5 activating monoclonal antibodies with chemotherapeutic agents that likely activate p53 and may inappropriately cause the loss of normal testicular germ cells and subsequent male infertility.

4.2 Results

A. TRAIL- and DR5-gene deficient mice show no phenotypic alterations in testicular morphology

Mice with a gene deficiency in either DR5 or TRAIL (**Fig. 4.1**, panel A) failed to show any morphological differences in the spermatogenesis of adult testis as compared to the matched wild type strain (**Fig 4.1**, panel B). No changes were observed in the fecundity or fertility of these mice as compared to the wild type background strain (data not shown).

B. TRAIL induces apoptosis in primary rat testicular germ cells

Preparations of rat testicular seminiferous tubule explants were used to test if exogenously added TRAIL (0.5 µg/ml) could trigger apoptosis of primary germ cells. This experimental approach allows for the preservation of the structural associations between the Sertoli cells and the germ cells. The total number of apoptotic cells per 1 mm segment was measured using the TUNEL assay on squash preparations as described by Toppari and Parvinin (Toppari, 1985). The number of apoptotic cells in control explants was variable (90 ± 40) and reflects the normal experiment-to-experiment variability in the handling and dissection of the tubule segments. (**Fig. 4.2**) Addition of the caspase-3 inhibitor DEVD (100 µM) to the media at the time of preparation caused a trend for lower baseline apoptosis values (60 ± 30). Addition of 0.5 µg/ml TRAIL induced a significant (*P*-value: ≤ 0.01) increase in the number of apoptotic germ cells per 1 mm tubule segment (260 ± 80), and this robust increase could be inhibited by pre-incubation with DEVD.

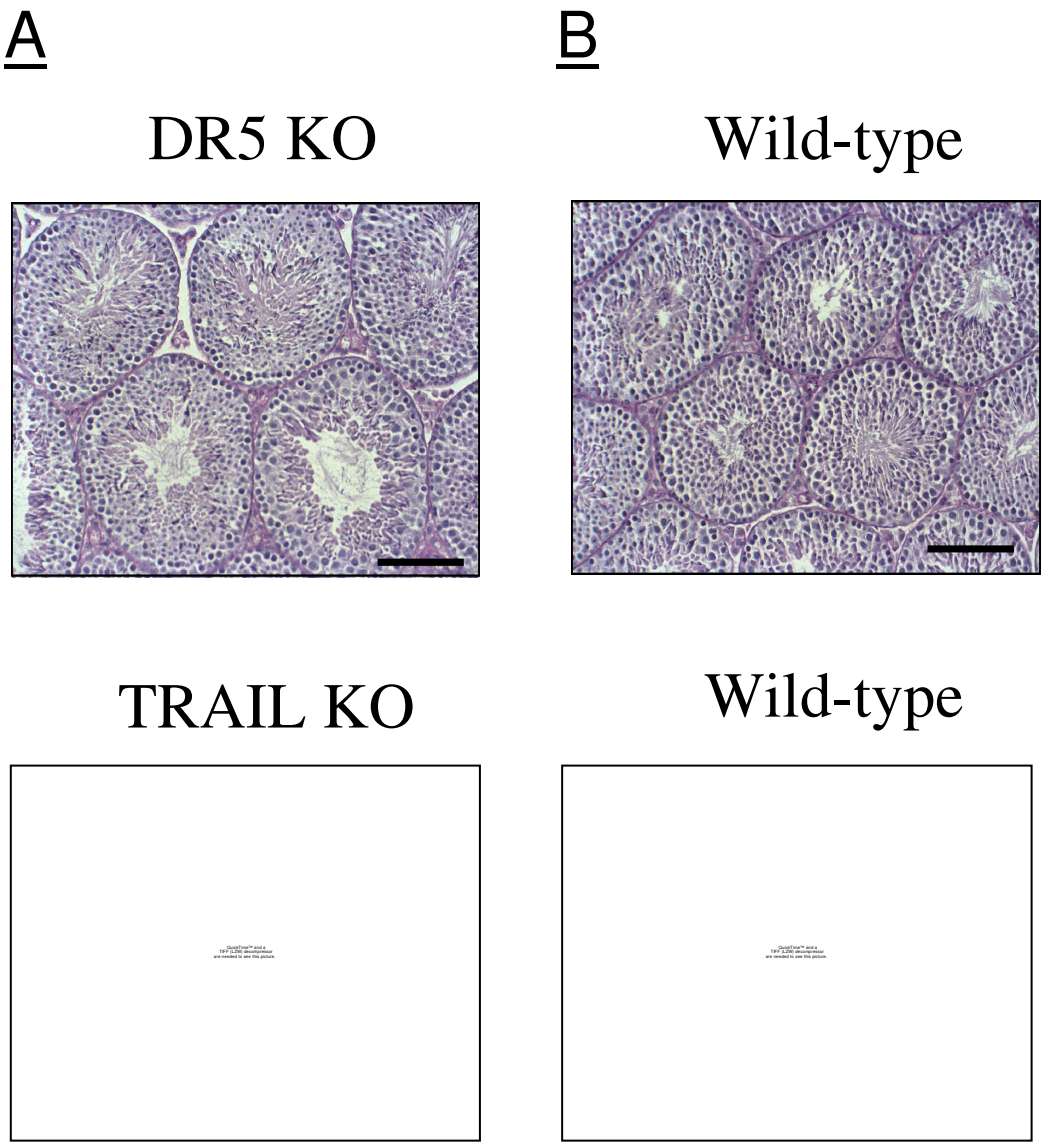

Fig. 4.1. *Testicular histology of wild type mice versus TRAIL deficient and DR5 deficient mice.* Cross sections of 5 µM thick tissue were treated with PAS & H as described in the *Methods and Materials*. Neither TRAIL- nor DR5-gene deficient mice exhibit significant morphological changes under physiological conditions. The gene deficient animals were matched with normal testes of animals of the same stage and background. Bar equal to 135 µm.

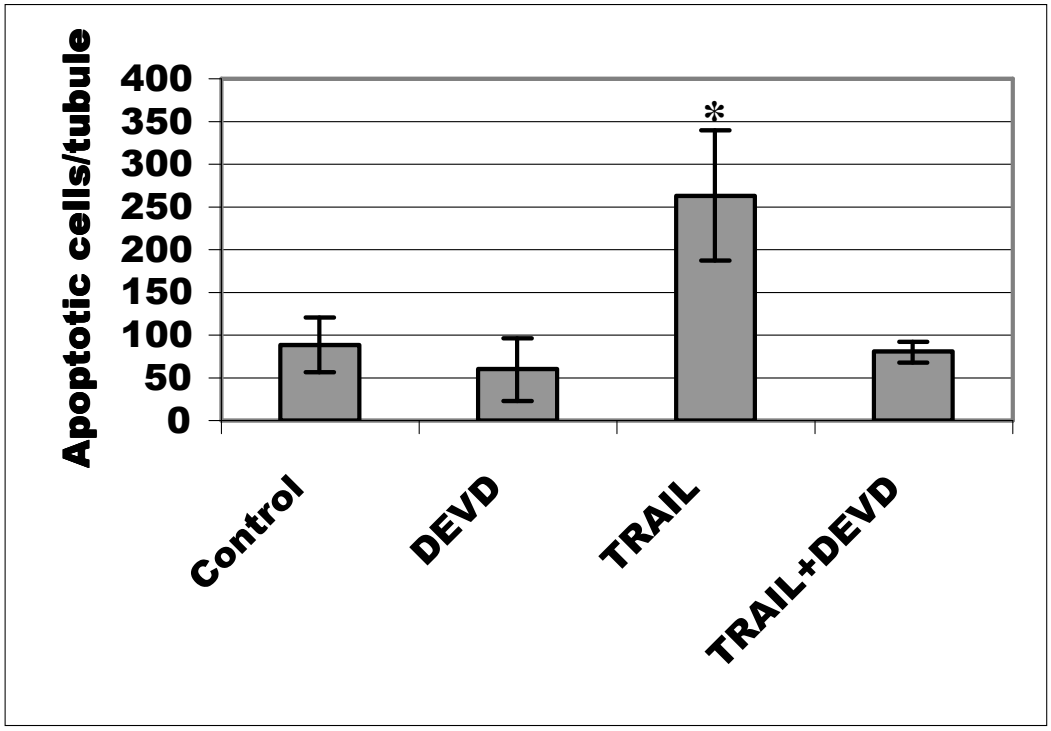

Fig. 4.2 *Recombinant TRAIL-induced apoptosis of primary rat testicular germ cells.* Segments of rat seminiferous tubules (1 mm) were isolated and maintained under serum free conditions as described in *Material and Methods*. TRAIL (0.5 µg/ml) was added to the media and germ cell apoptosis was assessed after 6 h by the TUNEL assay on squash preparations. Methyl green was used as a counterstain. For some experiments isolated tubule segments were pretreated with 100 µM z-DEVD-FMK for 1 h before TRAIL addition. *Samples are within standard error (*P*-value: ≤ 0.01) compared to controls as determined by analysis of variance (ANOVA) and Fisher's PLDS tests.

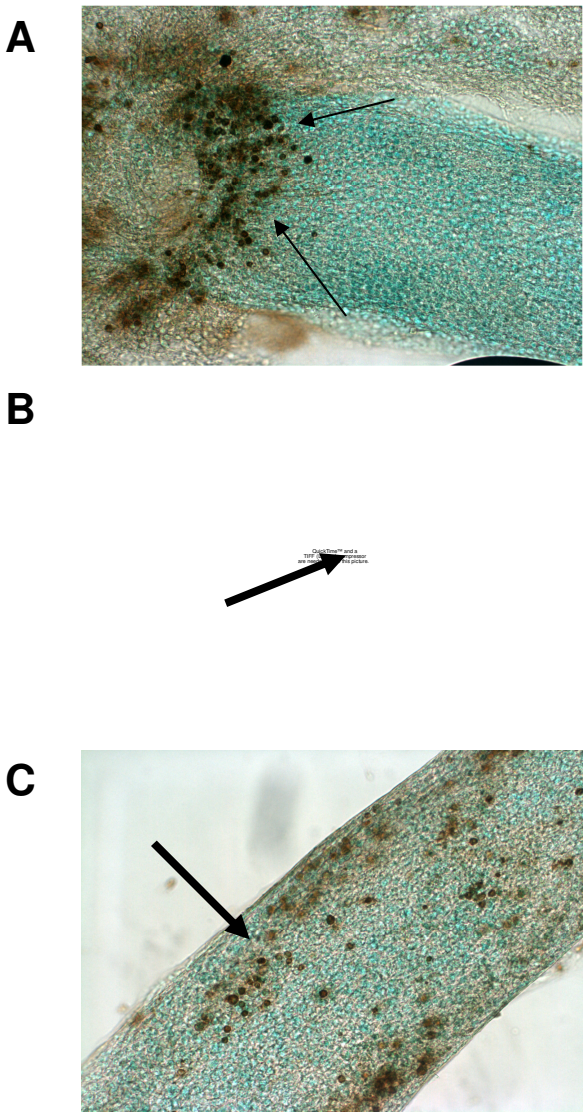

Fig. 4.3 *MD5-1 increases apoptosis in rat seminiferous tubule explants.* Explants from rats treated with TRAIL induced apoptosis after 6 h as detected by TUNEL. A combination of TRAIL (0.5 µg/mL) and DR5 agonistic antibody MD5-1 (0.5 µg/mL) stimulated apoptosis at a higher rate than TRAIL alone.

C. DR5 agonistic antibody MD5-1 increases apoptosis in rat seminiferous tubule explants

To determine whether a combination of TRAIL and a DR5 agonist would sensitize germ cells to greater apoptosis, the anti-DR5 antibody MD5-1 was administered. Primary rat seminiferous tubule cultures were again used as a model and cultured for 10 h prior to toxicant treatments. In the control explants, cell death is observed only in the exposed "ends" of the tubules, where germ cells no longer have contact with Sertoli cells (**Fig. 4.3A**, control panel). The body maintains proper structural relationships between the cell types and stains positive for methyl green but not for apoptosis. Treatments of TRAIL (0.5 μg/mL) alone (6 h) induces positive stain dispersed among the germ cells of the tubule (**Fig. 4.3B**, TRAIL). Explants exposed to a combined treatment of TRAIL and MD5-1 (6 h, 0.5 μg/mL) displayed extensively increased apoptosis throughout the body of the tubule (**Fig. 4.3C**, TRAIL + MD5-1). The tubules were partially protected from apoptosis when pre-treated (1 h) with caspase-3 inhibitor, DEVD (not shown).

D. TRAIL treatments induced apoptosis in p53 expressing GC-2 cells *in vitro*

Treatments of TRAIL modestly engages the apoptotic program in GC-2 spermatocyte-like cultured cells as determined by annexin-PI flow cytometry. Cell death is detected at 6 h time points in p53 permissive cells maintained at 32°C (**Fig. 4.4A**). At non-permissive temperatures (37°C) exogenously added TRAIL had little effect on the cells and did not produce significant apoptosis (data not shown). Initial concentrations of .25 μg/uL produced apoptosis at twice the rate of the control and were significant (*P*-value: ≤ 0.01) compared to the control (untreated) sample. A further concentration of .50 μg/uL further increased cell death to 21%, approximately what was obtained when using the Fas-linker Jo2 in GC-2 cells (**Fig. 3.4A**).

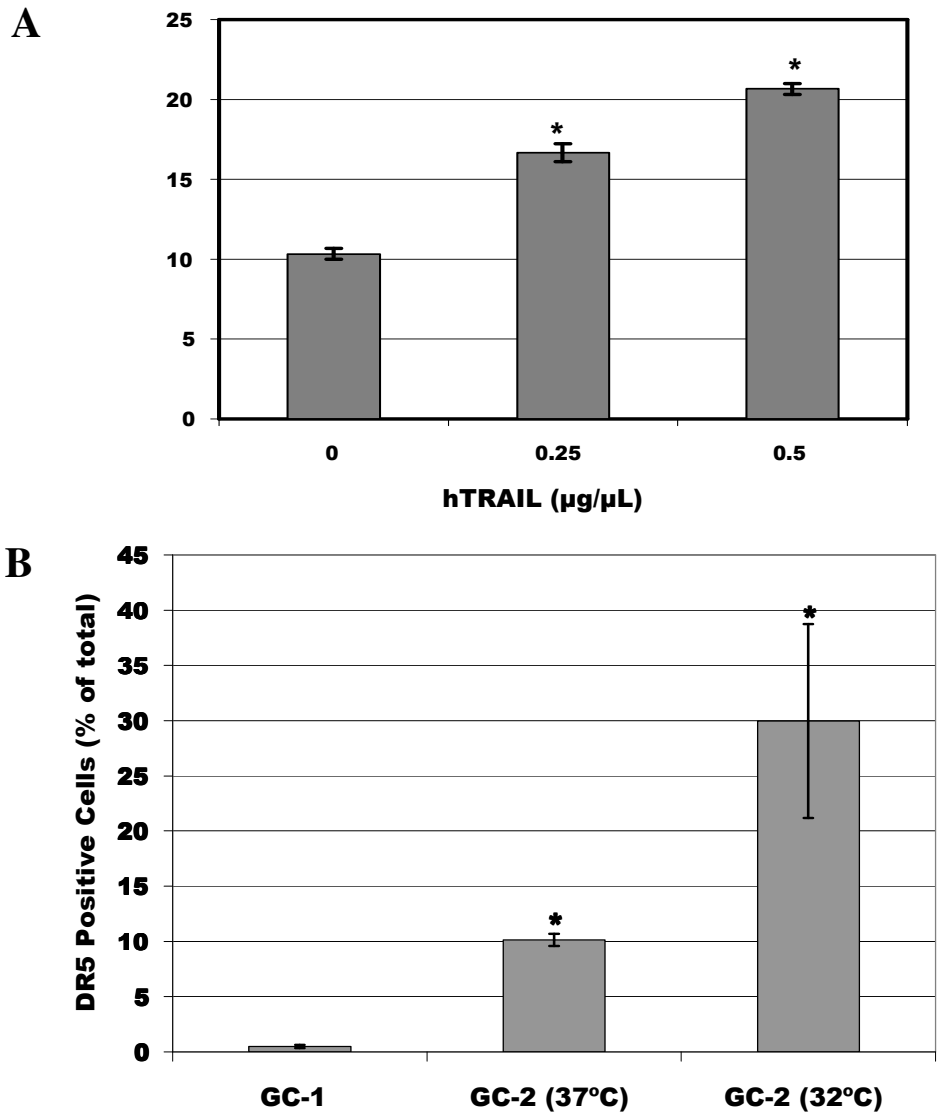

Fig. 4.4 *TRAIL induces apoptosis in GC-2 cells.* **(A)** Treatments of TRAIL modestly engages the apoptotic program in GC-2 spermatocyte-like cultured cells as determined by annexin-PI flow cytometry. Cell death is detected by annexin-PI flow cytometry at 6 h time points in p53 permissive cells maintained at 32° C. Experiment performed by Yang Ye. **(B)** *Membrane DR5 protein elevated in p53 permissive GC-2 cells.* The level of expression of DR5 on the cell membrane were determined by surface antigen flow cytometry. *Samples are within standard error (*P*-value: ≤ 0.01) compared to controls as determined by analysis of variance (ANOVA) and Fisher's PLDS tests.

E. Cell surface expression of DR5 greater in p53-expressing GC-2 cell lines

The expression of DR5 on the plasma membrane of GC-1 and GC-2 cells (maintained at either 37°C or 32°C) was determined by surface antigen flow cytometry. GC-1 cells are of a spermatogonia lineage and are used as a control to GC-2-spermatocyte cultures. Only a small percentage (<1%) of GC-1 cells revealed positive cell surface expression for DR5 (**Fig. 4.4B**). However, 10% of the GC-2 cell population maintained at 37°C were positive for DR5 expression whereas approximately 30% of GC-2 cells maintained at 32°C showed positive membrane expression.

F. Cyclohexamide pre-treatment does not significantly enhance GC-2 sensitivity to TRAIL-induced apoptosis

Pretreatment of cells with low doses of cyclohexamide has previously been used as a mechanism to sensitize certain cell types to death receptor-induced apoptosis (Wajant, 2000), (Kreuz, 2001). Exposure of GC-2 cells (32°C) to cyclohexamide for 6 h was found to increase the incidence of cell apoptosis (**Fig 4.5A & B**). GC-2 cells that are undergoing apoptosis are easy to discern in cell culture due to their loss of adhesion with the matrix on the plate and their rounding morphology (**Fig. 4.5A**). Pretreatment of GC-2 cells maintained at 32°C with cyclohexamide and subsequent treatment with TRAIL (0.5 μg/ml) gave an additive increase in cell apoptosis indicating that these two agents likely instigate apoptosis by two distinct mechanisms. As expected, treatment with cyclohexamide, TRAIL, or the combination did not increase apoptosis in GC-1 cells (**Fig. 4.5B**, no photomicrograph shown). The lack of cell death in GC-1 cells can therefore be at least partially due to low level of DR5 expressed in spermatogonia-like cells.

G. DR5 agonistic antibody (MD5-1) pretreatment results in a robust synergistic increase in TRAIL-induced apoptosis in GC-2 cells

The MD5-1 anti-DR5 monoclonal antibody was originally produced in the laboratory of Dr. Hideo Yagita of the Juntendo University School of Medicine in Tokyo, Japan. It was reported that this antibody exhibited potent anti-tumor effects against TRAIL-sensitive tumor cells in mice (Takeda, 2004). Preliminary studies in our laboratory using MD5-1 revealed that pretreatment with this antibody caused the GC-2 cells maintained at 32°C to be extremely sensitive to TRAIL-mediated apoptosis and required an earlier 2 h time period for analysis of germ cell apoptosis. After 2 h of TRAIL exposure of GC-2 cells only a slight increase in apoptosis was measured and this could be reduced by exposure to the pan-caspase inhibitor z-VAD-fmk (**Fig. 4.6A**, panel a-c). Treatment of GC-2 cells with anti-DR5 alone did not increase apoptosis but, in combination with TRAIL, induced substantial amounts of apoptosis and this cell death could be inhibited by concurrent z-VAD-fmk treatment (**Fig. 4.6A**, panels d-f). Quantitation of GC-2 cell apoptosis shows that nearly 60% of cells that had been pre-treated with anti-DR5 antibody had undergone apoptosis by 2 hour after TRAIL treatment whereas those treated with TRAIL or anti-DR5 alone did not show significant increases in apoptosis (**Fig. 4.6B**). Partial protection could be seen when caspase inhibitor ZVAD was used to block the effect, indicating caspase dependent apoptosis. However, the inhibitor was not enough to completely abrogate the effect of MD5-1/TRAIL treatments, which initiates cell death within 30 minutes.

Western blotting shows that the MD5-1/TRAIL co-treatment results in levels of reduced full-length caspase-8 after treatment and particularly at the p53 permissive temperature, indicating its activation. (**Fig. 4.6C**) Associated cleavage and activation of caspase-3 also occurs post treatment. These findings supplement previous observations that p53 activity is associated with increased caspase activation and that TRAIL/MD5-1 treatments induce the extrinsic pathway in spermatocytes.

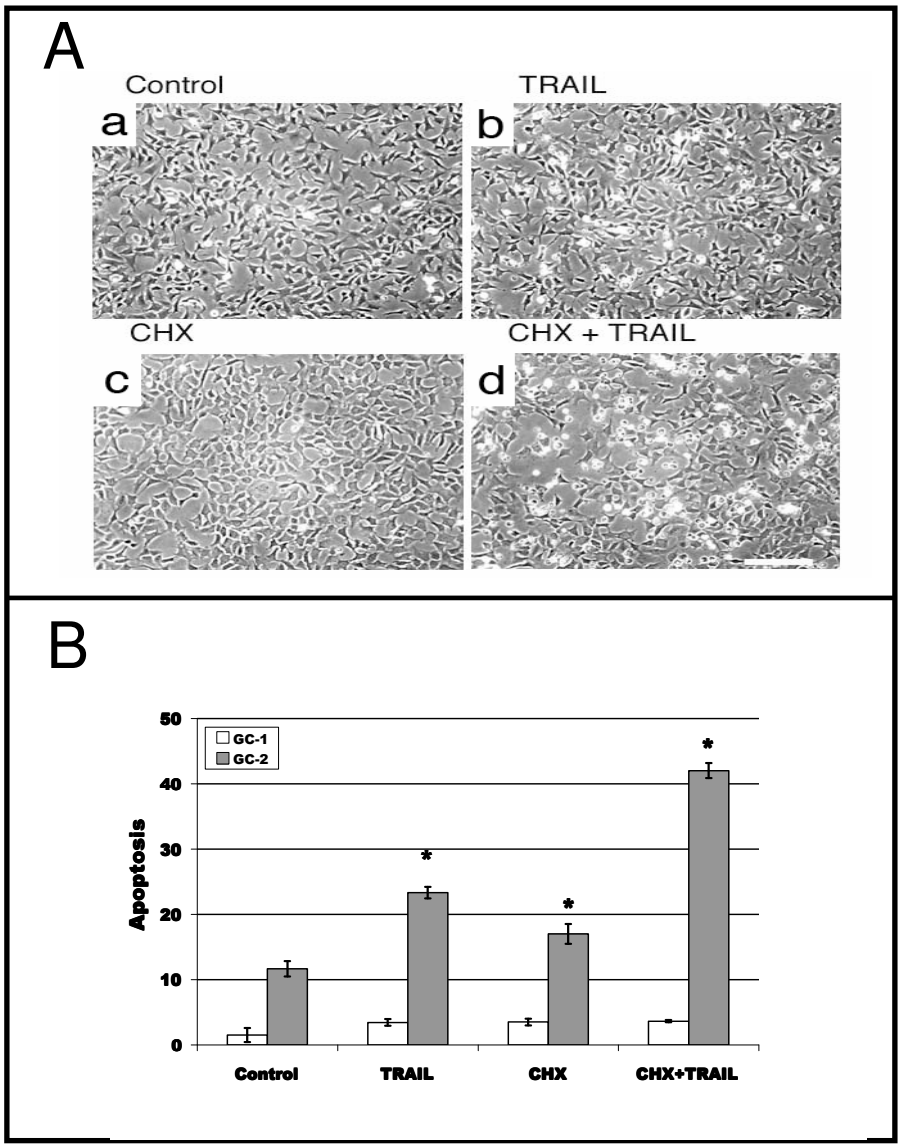

Fig. 4.5 *Cyclohexamide (CHX) pretreatment causes a modest sensitization of TRAIL-induced cell death in GC-2 (32°C) cells.* (**Panel A**) Phase-contrast micrographs of GC-2 cells cultured at 32 °C. Cells were treated with either vehicle (a), TRAIL (0.5 µg/ml) (b), CHX (1 µg/ml) (c), or a 1 h pre-treatment with CHX before the addition of TRAIL (d). Bar equals 100 µM. (**Panel B**) GC1 and GC-2 (32°C) cells were treated with vehicle, CHX, TRAIL or the combination or CHX and TRAIL as described above and cell apoptosis was evaluated after 6 h of exposure *via* annexin-PI flow cytometric analysis. *Samples are within standard error (*P*-value: ≤ 0.01) compared to controls as determined by analysis of variance (ANOVA) and Fisher's PLDS tests.

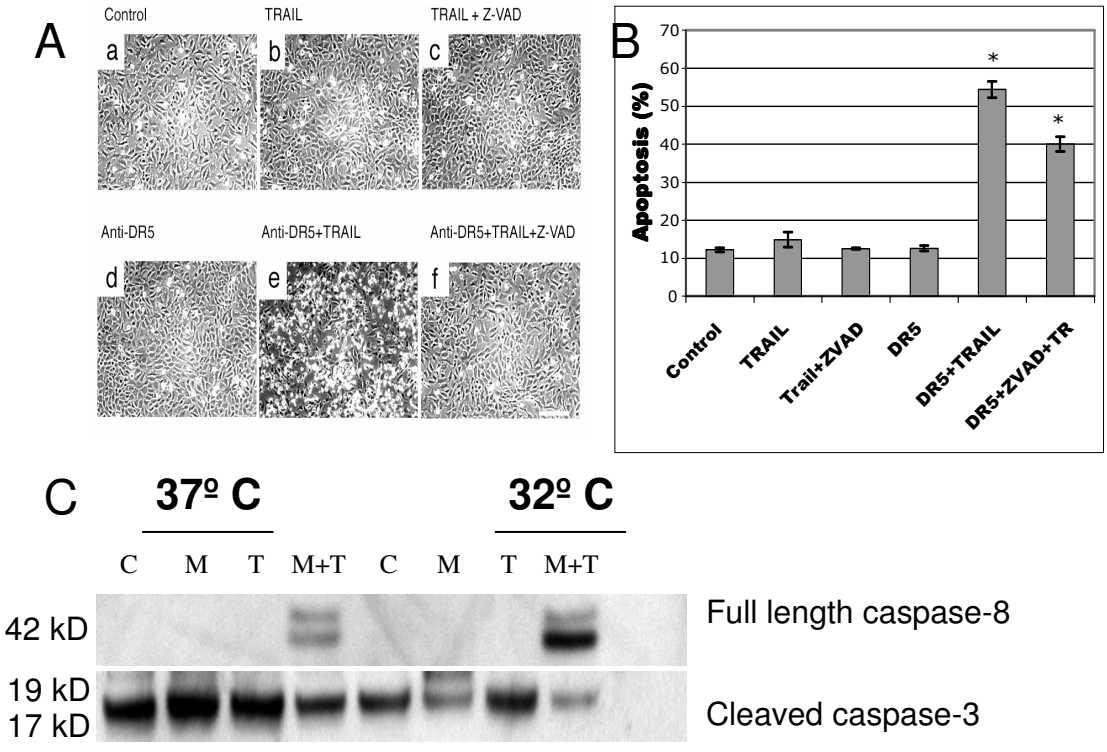

Fig. 4.6 *TRAIL-induced apoptosis of GC-2 (32°C) cells is significantly increased after pretreatment with an anti-DR5 antibody.* (**Panel A**) Phase-contrast micrographs of GC-2 cells maintained in culture at 32°C. Cells were treated with either vehicle (a), TRAIL (0.5 µg/ml) (b), z-VAD-fmk-FMK (10 µM) and TRAIL (c), agonistic anti-mouse DR5 (MD5-1) antibody (0.5 µg/ml) (d), the combination of anti-DR5 antibody and TRAIL (e), or a 1 h pretreatment with z-VAD-fmk-FMK (10 µM) before the addition of TRAIL and anti-DR5 antibody (f). The images of the cells in culture were captured after 2 h of exposure. Bar equals 100 µM. (**Panel B**) GC-2 (32°C) cells were treated with vehicle, TRAIL, anti-DR5 antibody or the combination of the anti-DR5 antibody and TRAIL as described above and cell apoptosis was evaluated after 2 h of exposure *via* annexin-PI flow cytometric analysis. *TRAIL/MD5-1 treatments preferentially induce caspase-8.* Treatment of GC-2 cells with Trail (.5 µg) and MD5-1 (.5 µg) induces high levels of apoptosis and activated caspase-8 after only 2 h incubations. *Samples are within standard error (*P*-value: $\leq$ 0.01) compared to controls as determined by analysis of variance (ANOVA) and Fisher's PLDS tests.

H. Anti-DR5 antibody and p53 activity causes stabilization/clustering of DR5 on the cell membrane and increased sensitivity to TRAIL

Addition of MD5-1 generates cell death in cells grown both at 37° (data not shown) and 32° C. Apoptosis is much greater in the cells maintained at 32°C where p53 activation and nuclear localization occurs (**Fig. 4.6 A & B**). Immunocytochemical staining and confocal microscopy of DR5 in the 37° sample (**Fig. 4.7A**; limited p53 expression and no MD5-1 treatment) is light and diffused randomly about the membrane surface. Further evaluation demonstrated that an increase in the p53 activation in GC-2 cells correlated with higher DR5 levels on the membrane at 32°C (**Fig. 4.7C**). Treatments with MD5-1 promote the aggregation and organization of DR5 receptor proteins into denser regions along the surface of the plasma membrane, even in cells that express less p53 (**Fig. 4.7B**). However, elevated p53 expression in the cells maintained at 32°C increases DR5 localization to the membrane and GC-2 cells become notably more sensitive to MD5-1 treatment and subsequent TRAIL addition (**Fig. 4.7D**). Cells exposed to this combined treatment were rapidly killed after only a 2 h treatment course, with the initiation of apoptosis occurring as early as 30 min. post administration.

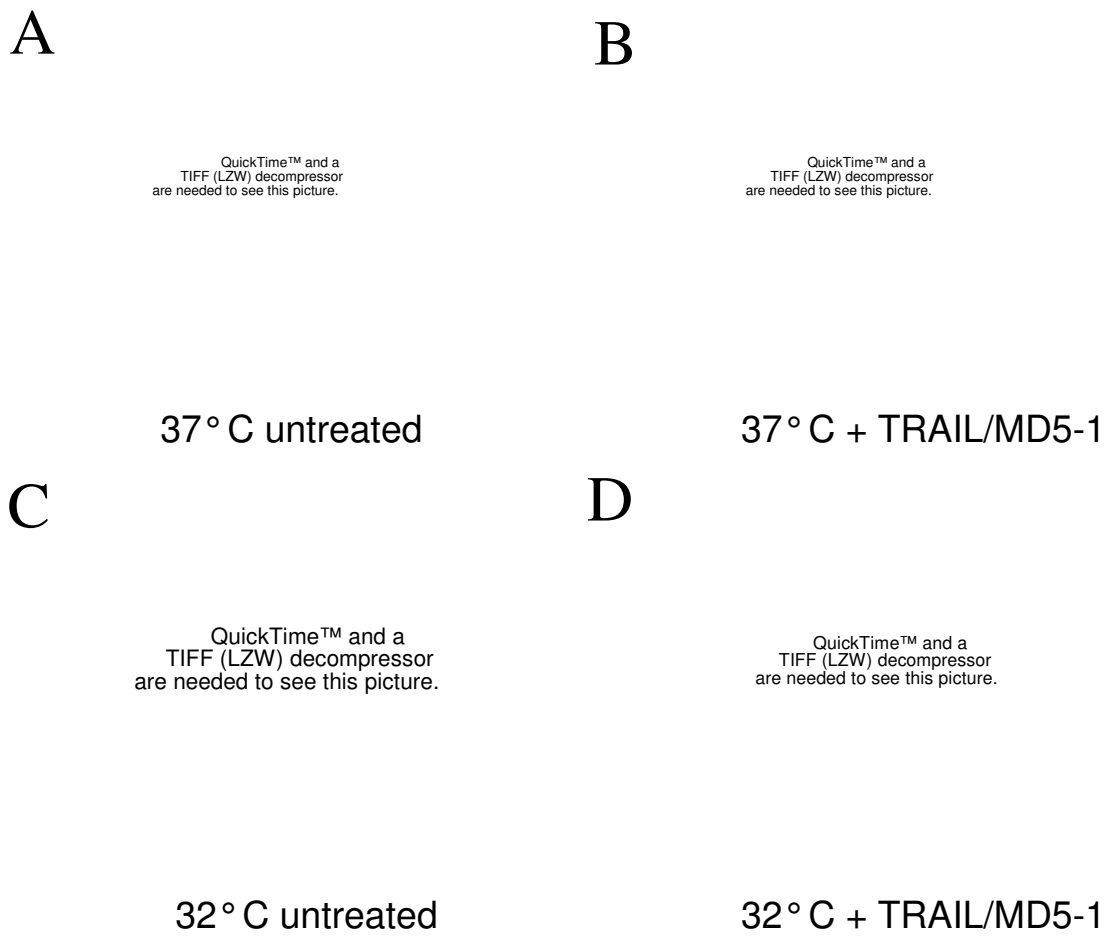

Fig. 4.7. *DR5 agonist antibody MD5-1 and p53 are able to increase and cluster DR5 on the membrane of GC-2 cells.* Immunocytochemistry of GC-2 cells grown at both p53 permissive (32ºC) and non-permissive (37ºC) temperatures. Cells were grown on chamber slides for 24 h before detection with an anti-DR5 primary antibody and Alexa Fluor 488 secondary antibody. Treatments were conducted for two hours. Slides were mounted using Vector mount containing PI to stain the nucleus and all samples were analyzed *via* confocal microscopy. **Panel A** shows cells grown at 37ºC without treatment while **panel B** indicates cells at 37ºC after treatment with anti-DR5 antibody MD5-1 and TRAIL. GC-2 cells were also gown at the p53 permissive temperature without any treatment (**panel C**) or with MD5-1 antibody and TRAIL (**panel D**).

4.3. Discussion

The ability of TRAIL to trigger apoptosis in non-tumorigenic cells is rare. Hepatocytes appear to be a major exception to this rule (Lawrence, 2001), (Mori, 2004). In addition, resting lymphocytes may be also susceptible to TRAIL-mediated apoptosis under some physiological conditions (Meurette, 2006). However, normal germ cells have never been shown to undergo apoptosis though this pathway. In this chapter, evidence is presented showing primary germ cells explants undergoing cell death mediated by addition of human TRAIL. Furthermore, in GC-2 cells TRAIL-mediated apoptosis is further mitigated by p53 activity and is dramatically augmented by the DR5 agonistic antibody, MD5-1.

Seminiferous tubule explants represent a 1 mm length of testicular cells removed from the testis and cultured for a short time (10 h) in media. The use of explants allows for the easy delivery of agents to the cells of the seminiferous tubule without instigating a disruption of the intimate associations between Sertoli cells and germ cells. Addition of exogenous recombinant TRAIL (0.5 μg/mL) protein was able to induce a 3-fold elevation in germ cell apoptosis (**Fig. 4.1**). The TRAIL mediated germ cell apoptosis was significantly inhibited by the caspase-3 inhibitor DEVD (**Fig. 4.1**) demonstrating its dependence on caspase activation. This is the first time TRAIL has been shown to trigger cell death in primary testicular cells. Although TRAIL was able to cause increases in germ cell apoptosis in this *in vitro* tissue model, direct injection of TRAIL into the testis was not found to cause increases in germ cell apoptosis (data not shown). These findings may indicate that TRAIL may only be able to instigate apoptosis in germ cells that have undergone secondary changes due to cellular stress, such as that which occurs during the preparation of the tissue explants.

In the testis, apoptosis of germ cells normally occurs under physiological conditions and is speculated to control the numbers of germ cells to match the supportive capacity of the testis (Print, 2000). In previous studies, the functional participation of another death receptor ligand system, the FasL/Fas system, has

been shown to induce testicular germ cell apoptosis in rodents after exposure to the Sertoli cell toxicant MEHP. A functional FasL/Fas signaling system, however, does not appear to be a requirement for physiological apoptosis in the testis as mice expressing mutant forms of these proteins or with a gene deficiency are still fertile and show normal spermatogenesis.

Similar to these observations, the mice that are gene deficient for TRAIL or DR5 show apparently normal levels of fertility (unpublished observations from our mouse colony). In addition, mice deficient for either TRAIL or DR5 do not exhibit unusual levels of apoptosis or display morphological derangement under physiological conditions (**Fig 4.2**, panels A & B). Therefore, TRAIL/DR5 induced apoptosis of testicular germ cells is not an absolute requirement for the physiologic control of germ cell apoptosis in the testis. Howerver, TRAIL and DR5 are kown to be expressed at a high rate in germ cells (Grataroli, 2002), (Giammona, 2002). Taken together with our present findings indicating that TRAIL induces germ cell death, this has led us to hypothesize that alterations in germ cells occur only after distinct testicular stress/injury. Our finding that TRAIL was only able to induce apoptosis in explants of testicular seminiferous tubules and not in the intact testis *in vivo* is in agreement with this hypothesis.

An exciting additional find was that the combination of TRAIL and DR5 agonistic antibody (MD5-1) resulted in a much higher apoptotic response in primary tubule cultures than just TRAIL alone (**Fig. 4.3B**). Ordinarily, healthy tubules will only demonstrate apoptosis on the exposed "ends," (small arrows) whose germ cells have lost contact with the supporting Sertoli cell **(Fig. 4.3A**) Eventually, these cells die from lack of nutrients or some other stress related to Sertoli cell dissociation. However, the body of the tubule maintains Sertoli cell-germ cell structural integrity and no apoptosis occurs in untreated cultures. Addition of 0.5µg/ml of TRAIL increases the incidence of germ cell death in the tubule body (large arrows). Combined treatment with both TRAIL and MD5-1 significantly increases this cell death and apoptotic positive germ cells can be seen throughout the tubule (**Fig. 4.3C**).

In order to discern the cellular determinants of germ cell sensitivity to TRAIL we utilized germ cell-derived cell lines GC-1 (spermatogonia-like) and GC-2 (spermatocyte-like). Initial experiments determined that human TRAIL sensitized p53 permissive GC-2 cells to moderate apoptosis (**Fig. 4.4A**). We have previously demonstrated that GC-2 are differentially sensitive to Fas-induced cell death coincident to their p53 status. Here we show that GC-2 cells maintained at the p53 permissive temperature exhibit the highest membrane DR5 levels (30%) among the cells examined, as determined by flow cytometry (**Fig. 4.4B**). Both the spermatogonia-like GC1 cells and GC-2 cells maintained at the non-permissive p53 temperature expressed considerably less DR5 protein on their cell membranes (<1% and 10% expression respectively). These differences in membrane DR5 expression correlate well with their sensitivity to TRAIL-mediated apoptosis, indicating that the sensitivity of the spermatocytic population of germ cells is closely associated with receptor expression on the cell membrane.

The use of antibodies directed against the TRAIL receptors DR4 or DR5 as strategies to treat cancer has been previously explored and a number of groups have specifically used DR5-specific monoclonal antibodies (Ichikawa, 2001), (Guo, 2005). Here we used the agonistic anti-DR5 monoclonal antibody MD5-1 to investigate its ability to induce DR5 mediated apoptosis in the GC-2 germ cell lines. The above experiments with tubule cultures indicated that germ cells were sensitive to apoptosis *via* the TRAIL/DR5 system and also that a caspase blocker (DEVD) would protect against cell death.

The *in vivo* results were validated in GC-2 cells. The results showed that although addition of TRAIL alone induces modest increases in cell death, addition of the MD5-1 was able to generate a robust synergistic increase in germ cell apoptosis (**Fig. 4.6A**). Treatment with MD5-1 elevated the incidence of germ cell apoptosis by four-fold within two hours of addition, resulting in an apoptotic incidence of nearly 60%, compared to the control, TRAIL only treatment, or with MD5-1 only (12, 15, and 13% respectively). We also show here that in cultured GC-2 cells treated with TRAIL and agonistic antibody, caspase activation of the

extrinsic pathway is induced, as seen by the robust reduction of full-length caspase-8. Additionally, induction of the effector caspase-3 is only observed in GC-2 cells treated with both TRAIL and the DR5 agonist, MD5-1. Caspase inhibitor z-VAD-fmk was able to at least partially block the effect of TRAIL/MD5-1 (**Fig 4.6B**).

These experiments contrasted with the six hour time points needed to observe significant cell death in the experiments using cyclohexamide (CHX), a protein translation inhibitor widely employed to sensitize cells to TRAIL-mediated apoptosis (Wajant, 2000), (Kreuz, 2001). Although TRAIL or cyclohexamide treatment alone was able to induce moderate increases in apoptosis in GC-2 cells maintained at the p53-permissive temperature (**Fig. 4.5B**), the combination of the two together did not result in a synergistic effect but rather a simple additive effect. This indicates that MD5-1 and cyclohexamide addition may induce apoptosis *via* independent mechanisms.

To explain the influence of MD5-1 on the sensitivity of DR5 to TRAIL activation we tested whether MD5-1 was simply acting to stabilize and/or cluster DR5 trimers on the cell membrane. Trimerization of DR5 receptors, as with Fas receptors, is required for efficient activation of procaspase-8 in the DISC (Schulze-Osthoff, 1998). Poor or low clustering of death receptor proteins impede the formation of the DISC at the membrane and therefore little catalytic cleavage of procaspse-8 into its active form. To assess this possibility we performed immunocytochemical analysis of DR5 in GC-2 cells at both the p53 permissive and non-permissive temperatures after treatment with MD5-1, TRAIL, or a combination of both. Visualization of the cells via confocal microscopy showed evidence that cells grown under p53-permissive conditions expressed a considerably increased level of DR5 on the cell membranes than did cells grown at 37°C (**Fig. 4.7A & C**). Cells grown at 37°C could also show elevations in DR5 on the membrane if followed with administration of MD5-1, although the receptor increases were not robust. The DR5-agonist potentially acts to cluster the smaller amount of receptor protein on the membranes of these cells. The combination of

p53 expression and MD5-1 (**Fig. 4.7D**) induced a highly elevated level of membrane DR5 and this likely results from both increased transcription of DR5.

For apoptosis to occur, DR5 levels are already present on the surface of the germ cell, likely pre-associated into trimers or clusters of trimers as Fas receptors are known to do. MD5-1 may act to aid in the clustering of multiple death receptor trimer sites. In favor of our hypothesis, the confocal microscopy results show not only more DR5 expression but tighter overall organization of the staining in p53 permissive cells. This effect has been previously seen in apoptotic cells with Fas receptor "capping" (Cremesti, 2001). Therefore, when TRAIL is added exogenously to the cells, the ligand is able to interact with "groups" of trimerized receptors and apoptotic induction can occur at a very rapid pace. In addition, the vigorous pro-death signal may displace the anti-apoptotic c-FLIP protein and generates high levels of apoptosis. These data, taken together, indicate that the expression of DR5 on the membrane of germ cells is positively associated with the transcriptional activity of p53 and that the anti-DR5 antibody is a strong sensitizer of membrane DR5 to TRAIL-induced apoptosis.

The current study is important because of its identification of defined germ cell sub-populations (spermatocytes) sensitive to specific apoptotic regulation and a method by which to sensitize this population to TRAIL/DR5 dependent cell death. In addition, p53 activation is critical in the induction of apoptosis both *in vivo* and *in vitro*. The data indicates that under physiological conditions TRAIL and DR5 do not cause germ cell death. Rather, cell stress associated with chemotherapeutic agents or toxicant exposures is required to induce the observed sensitivity. These findings have implications for current chemotherapeutic approaches that combine DR5 activating monoclonal antibodies with TRAIL treatments.

Chapter 5:
Regulation of Germ Cell Apoptosis by Ubiquitinylation

5.1. Introduction and Rationale

In the previous chapters, it has been shown that MEHP triggers the p53-dependent expression of Fas and DR5 and that the initiation of apoptosis occurs through the extrinsic pathway. However, regulatory barriers exist in the pathway to safeguard the cell against unimpeded apoptosis. One such element is the c-FLIP protein, a major determinant of cell survival in response to death receptor mediated apoptosis. The ratio of c-FLIP protein to procaspase-8 present at the DISC is an important indicator of whether the cell will survive or undergo cell death. If the balance is tipped towards caspase-8 activation and apoptosis, c-FLIP is thought to be degraded through the ubiquitin-proteasome pathway. However, to date, the specific proteins responsible for targeting c-FLIP for degradation in germ cell apoptosis is unknown. This chapter examines the possible participation of an E3-ligase containing protein, Itch, in the sensitivity of spermatocytes to undergo cell death.

The ubiquitin-proteasome system is the most common method of protein degradation in the cell. Ubiquitination is ATP dependent and requires three intermediate enzymes: E1 (transferring), E2 (conjugating), and E3 (ligating), with the E3 ligase determining protein specificity (Haglund, 2003). The process of degradation is mitigated through the deposition of individual ubiquitin molecules through a particular lysine residue and onto a targeted protein. Multiple additions of ubiquitin through the same amino acid will eventually produce a chain of four or more that signals the identified protein for degradation (Pickart, 2001).

There are two classes of E3 ligases: RING (really interesting new gene) and HECT (homology to E6-AP carboxyl terminus) (Sun, 2003). HECT E3 ligases directly transfer ubiquitin to the substrate molecule whereas RING assists its conjugate E2 enzyme in this transfer. However, in both classes the poly-

ubiquitinylated protein is directed to the 26s proteasome, a large cytosolic complex that is capable of proteolysis and regeneration of constitutive amino acids.

Numerous proteins intimately involved with the apoptotic process are regulated through ubiquitinylation, including but not limited to p53, several members of the caspase family, and various BCL-2 proteins (Yang, 2000), (Orlowski, 1999), (Yang, 2003). Despite mounting evidence that c-FLIP is post-transcriptionally controlled *via* a ubiquitin ligase, no candidate proteins were put forward until the E3 ligase activity of Itch was associated with increased sensitivity to cell death in mouse hepatocytes (Chang, 2006). Itch was subsequently shown to promote liver cell apoptosis induced by TNF-alpha, possibly as a consequence of specifically targeting c-FLIP for degradation. Itch is a multi-functional protein that contains a HECT E3 ligase. The protein has been previously implicated in the regulation of specific immunity, development, and most recently, apoptosis (Kostianovsky, 2007), (Di Marcotullio, 2006). An established line of Itch protein deficient mice, *Itchy*, has been frequently employed as an autoimmune disease model, as Itch influences T-cell differentiation and auto-immunity (Perry, 1998), (Matesic, 2006). Furthermore, the ligase has been shown to undergo activation by way of JNK1-mediated phosphorylation, on one or more of three amino acids: serine-199, threonine-222, and serine-232 (Gao, 2004).

Fas is a member of the TNF-alpha family of receptors and is an established inducer of apoptosis in a variety of cell types, including germ cells. In addition to recruiting and interacting with members of the DISC, expression of the death receptor has been shown to promote JNK1 activation, a MAP (mitogen activated protein) kinase also responsible for activating Itch (Toyoshima, 1997), (Schwabe, 2004). Therefore, induction of Fas may not only induce the DISC and caspase cleavage but also stimulate the downstream phosphorylation of Itch and its ability to ubiquitinylate specific protein targets. Following this reasoning, a hypothesis was advanced surmising that increases in Itch activation would occur

following treatments with MEHP, a known inducer of death receptors in germ cells. A further postulation is that the stabile expression of p53 would be necessary for the efficient induction of this process given the data obtained in the previous chapters.

We propose here, for the first time, that Itch may play a role in the regulation of c-FLIP and germ cell apoptosis. Occurrence of c-FLIP ubiquitinylation in the GC-2 cell line is shown to correlate strongly with the expression of p53 and in concert with apoptosis. Cell death has been observed in the testicular tissues of wild type mice after exposure to the toxicant MEHP. Here we show that cell death in spermatocytes is coincident with the activation of Itch and the reduction of c-FLIP. Furthermore, we show that Itch deficient mice express more c-FLIP than wild type strains. Administration of MEHP in *Itchy* does not significantly increase apoptosis in these mice and at least partially protects against the massive germ cell loss seen in treated wild type strains.

However, we also observe that *Itchy* animals display an increased incidence of apoptosis in a spermatocyte stage distinct from those killed by MEHP. In addition, blocking Itch activation or reducing cellular transcript levels *via* siRNA did not reduce apoptosis in GC-2 cells, suggesting that other functions in the cell are effected if Itch is reduced. Since the largest percentage of the cells undergoing apoptosis *in vivo* are in metaphase, Itch may play a role in the cell cycle. In support of this observation, *Itchy* mice maintain high levels of cyclin B1, a protein that must be degraded for the progression from metaphase to anaphase. Therefore, our investigations may have also uncovered a previously undescribed role that Itch plays in the succession of germ cell mitosis/meiosis.

5.2. Results

A. FLIP is present in the testis as the long isoform and is processed after MEHP treatment

Usually, c-FLIP exists in the cell as a long (56 kD) or short (28 kD) isoform. Western blots of testis from MEHP-treated mice reveal that the c-FLIP protein is expressed in the testis primarily as the long form, with little detection of the ~28 kD short isoform. When the long form c-FLIP is present at the DISC, it is cleaved once by caspase-8, forming a p43 fragment that remains attached to FADD (Scaffidi, 1999). Processing is most robust concomitant with p53 expression, as the p43 fragment is reduced in p53-/- mice (**Fig. 5.1**). Caspase-8 cleavage is evidently concurrent with these processed forms, suggesting that both procaspase-8 and c-$FLIP_L$ are located in the DISC simultaneously. However, full-length c-$FLIP_L$ is present in only moderate amounts in the control and is reduced in samples treated with MEHP at 12 and 24 h **(Fig. 5.1)**. The results indicate that c-$FLIP_L$ is a fast turn-over protein that can be down-regulated after MEHP exposure.

B. Ubiquitinylation of c-FLIP is increased following Fas triggering and p53 expression *in vitro*

To determine if c-$FLIP_L$ undergoes post-translational modification by ubiquitin, c-$FLIP_L$ was immunoprecipitated from lysates of GC-2 cells following exposure to the Fas-activating antibody, Jo2. Immunoprecipitated complexes were probed using the anti-c-FLIP rat monoclonal antibody or ubiquitin (see *Methods*). The complexes collected through this procedure, upon being analyzed by western blot analysis, showed that cells at the p53 permissive temperature (32° C) had more extensive ubiquitin-tagged c-$FLIP_L$ than cells at the non-permissive temperature (**Fig. 5.2A**). Polyubiquitinylated forms of c-$FLIP_L$ were revealed as

discrete bands with molecular weights of >60 kDa on the western blot that corresponded to the predicted mass of C-FLIP$_L$ plus ubiquitin (**Fig. 5.2A**). Polyubiquitinylated levels of c-FLIP$_L$ were low in GC-2 cells grown at the p53 non-permissive temperature, but detectable levels were observed after Jo2 addition (**Fig. 5.2A,** 37° C). Cells cultured at the p53 permissive temperature exhibited a higher basal level of ubiquitinylation, which also increased after Jo2 treatment (**Fig. 5.2,** 32° C). However, c-FLIP$_L$ was similarly expressed at the permissive temperature in the absence of Jo2 treatment. Thus, p53 expression is an important factor in the degradation of the protein. Four or more protein bands approximately 8.5 kDa apart in addition to the naïve c-FLIP$_L$ protein band were generated, corresponding to the sizes expected for ubiquitin-tagged c-FLIP$_L$ protein (**Fig. 5.2**).

To validate the results, c-FLIP immunocytochemistry was performed on GC-2 cells maintained under both growth conditions without Jo2. Fluorescent detection of the c-FLIP protein was evident at the p53 non-permissive temperature and localized to the cytoplasm (**Fig. 5.2B**, left). In contrast, pre-existing c-FLIP was much less pronounced, even without administration of Fas-linker Jo2, when grown at the p53 permissive temperature (**Fig. 5.2B**, right). Thus, a causal link can be established between the expression of p53 and the levels of c-FLIP in GC-2 cells.

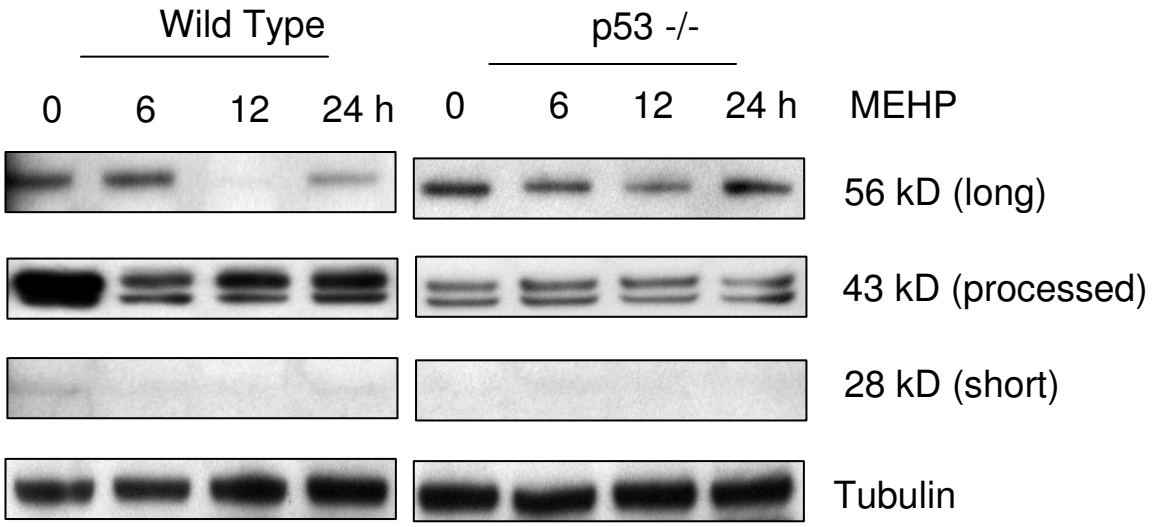

Fig. 5.1 *c-FLIP is expressed in testis primarily as the long form and is reduced after MEHP.* The long (56 kD) isoform of c-FLIP is reduced upon administration of MEHP in wild type mice but to a lesser extent in p53-/- animals. The presence of the c-$FLIP_L$ p43 processed form is also decreased in p53-/- mice. Very minor levels of short form (28 kD) were detected. Lack of p53 corresponds with less c-FLIP processing.

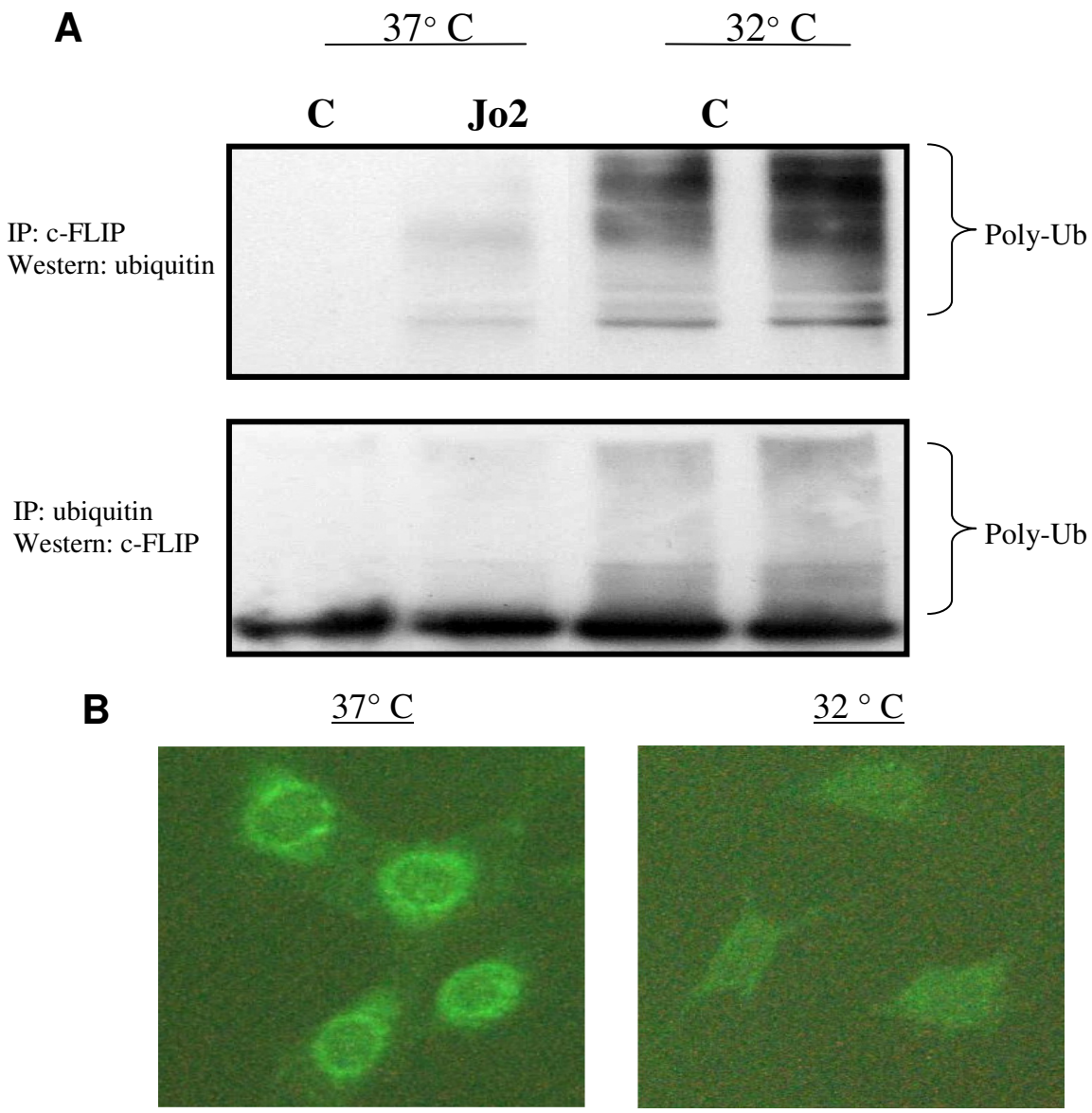

Fig. 5.2 *c-FLIP$_L$ ubiquitinylation in GC-2 cells is p53 dependent.* **(A)** Immunoprecipitation of GC-2 cells grown at non-permissive (37°C) and permissive (32°C) temperatures show a correlation between c-FLIP$_L$ ubiquitinylation and p53 expression. Pull-downs were performed in cell lysates as described in the *Methods and Materials*. Fas-linking antibody Jo2 (5 μg/ml) was used to supplement apoptosis but did not have a major influence. **(B)** Immunofluorescent detection of c-FLIP$_L$ in GC-2 cells at both temperatures. Cytoplasmic levels of c-FLIP$_L$ are decreased when p53 is expressed.

C. c-FLIP$_L$ levels are reduced in the presence of activated Itch *in vivo*

Whole testis from MEHP-treated wild type and p53-/- mice were homogenized and analyzed as described previously. Total Itch protein was expressed in the testis of both mouse strains and under all treatment conditions. Itch levels seem to decrease with MEHP exposure, perhaps correlating with its increased phosphorylation. To determine if Itch was activated at the same time points, an antibody against phospho-threonine-222 was used on the same samples. Threonine-222 is one of three amino acid residues (ser-199, thr-222, ser-232) that are commonly phosphorylated upon Itch activation (Gao, 2004). A steady increase in Itch activation was observed until very high levels were detected by 24 hours (**Fig. 5.3A**). This may explain why total Itch detection decreases, as the antibody detects a region (between amino acids 114-230) of the protein known to be phosphorylated. Additionally, the Itch activity corresponds to the activation of JNK1, a MAP kinase believed to the principle regulator of Itch phosphorylation. Interestingly, phospho-JNK 2/3 also increased slightly and are expressed at high levels. Correspondingly, c-FLIP$_L$ levels were reduced during the same time points as Itch/JNK1 were active in the wild type mice (**Fig. 5.3A,** left). The data suggests that decline of c-FLIP$_L$ protein and activation of Itch could be inversely proportional.

To assess whether the reaction was p53 dependent, p53-/- mice were also treated with MEHP (**Fig. 5.3A,** right). Itch is expressed in a uniform fashion regardless of treatment time points. Itch phospho-threonine-222 levels increased slightly but with less significance compared to the wild type mice. Likewise, c-FLIP$_L$ levels were largely unchanged and support the previously obtained GC-2 cell culture data in which p53 activation promotes c-FLIP$_L$ degradation. c-FLIP$_L$ is more highly expressed in the p53 deficient tissues than in comparable wild type samples, implying that p53 is an influence on the stability of c-FLIP$_L$ protein *in vivo* as well and that removing the protein confers a level of protection against its

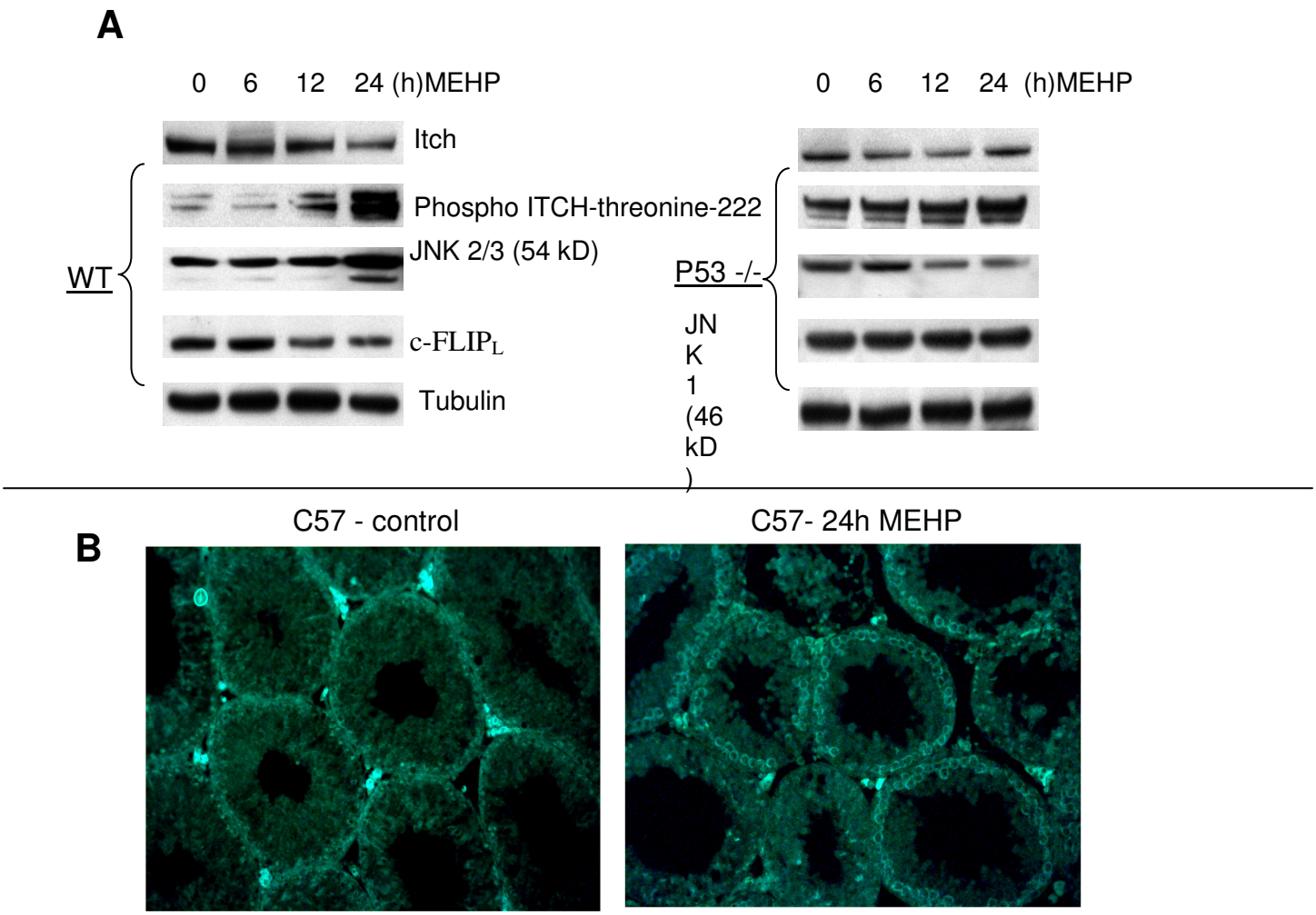

Fig5.3 *MEHP-mediated c-FLIP reduction p53-dependent and Itch associated.* **(A)** Wild type mice display a correlation between the activation of Itch and MEHP treatments. The activation correlates with increases in the MAP kinases JNK1/2/3 and a decrease in c-$FLIP_L$. In p53 deficient mice, Itch activation is lessened in response to MEHP and c-$FLIP_L$ levels are stabile. **(B)** Itch is localized to the cytoplasm of spermatocyte germ cell types following MEHP treatment.

MEHP-mediated degradation. Interestingly, no JNK1 was detected in p53 deficient mice, but JNK2/3 levels remained high.

D. Itch protein is increased and localized to the cytoplasm following MEHP treatment

Wild type mouse testis display a low but detectible level of Itch protein as determined by fluorescent immunohistochemistry. Mice exposed to MEHP for 24 hours stained strongly positive for Itch, particularly within the cytoplasm of spermatocytes (**Fig. 5.3B**). Itch protein localization to the spermatocyte at this later time point is consistent with increases in p53, death receptor expression, and apoptosis shown in the previous chapters.

E. Morphological staining shows partial protection from MEHP in *Itchy* mice

Morphological staining with periodic acid-Schiff's/hemotoxylin staining showed considerable germ cell loss, vacuolization, and Sertoli cell damage associated with MEHP treatment when adult wild type (**Fig. 5.4**, panel **A**) mice were treated at a 12 hour time point, consistent with previous results (**Fig. 3.1**). Mice lacking the functional *Itch* gene showed a moderate reduction in germ cells under control conditions, particularly spermatocytes and round spermatids, but were otherwise morphologically normal (**Fig. 5.4**, panel **C**). *Itchy* mice treated over a similar time course as the wild type displayed increased average lumen sizes, suggesting Sertoli cell cytoplasm reduction. However, the tubules of these mice displayed less damage structurally overall than the wild type samples and germ cell loss is minimal (**Fig 5.4**, panel **D**), suggesting that Itch removal confers at least partial protection against MEHP-mediated apoptosis.

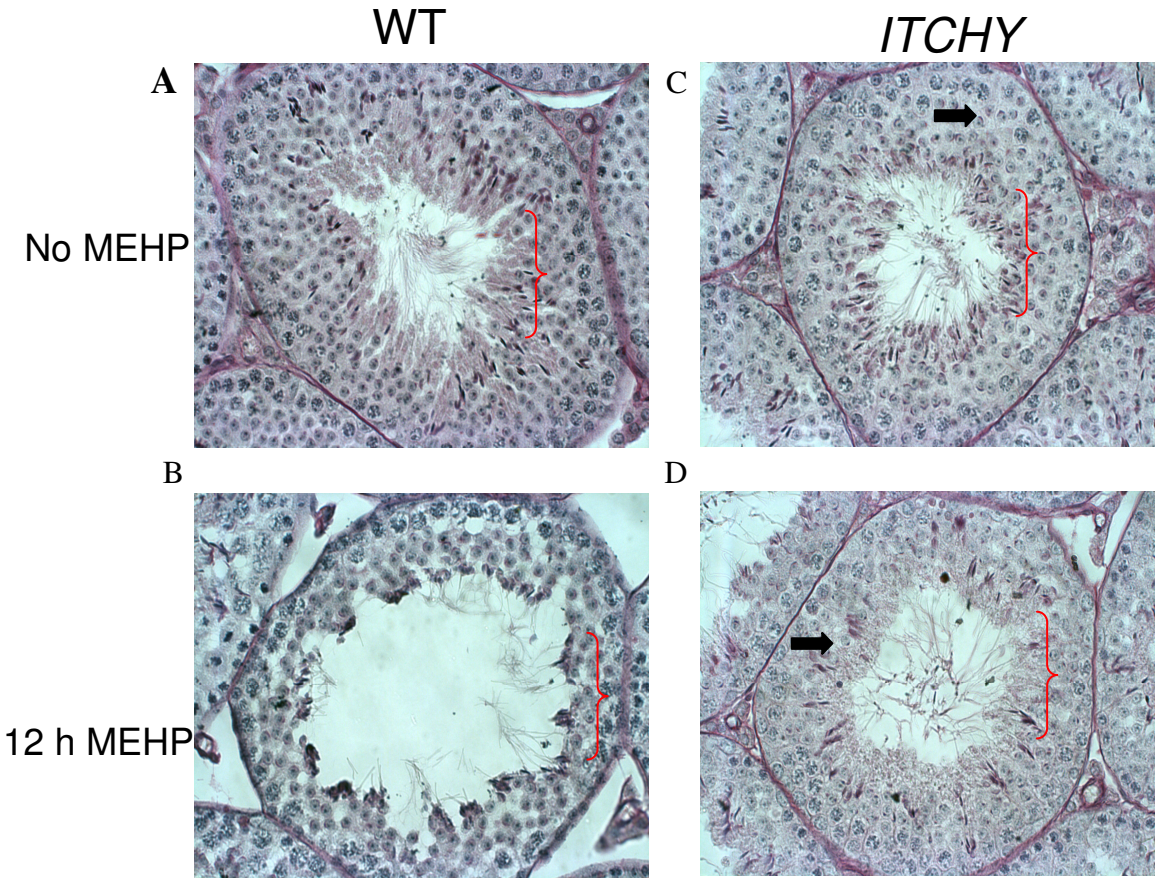

Fig. 5.4 *Itchy mice are partially protected from MEHP treatments.* PAS/H stained paraffin-embedded tissue shows severe morphological changes in MEHP treated WT mice at 12 h **(B)**. *Itchy* mice **(C)** show decreases in total germ cell numbers denoted by larger lumen size and in particular the loss of round spermatids but are otherwise morphologically similar to the wild type tubule **(A)**. *Itchy* mice maintain better structural integrity and exhibit less vacuolization after MEHP **(D)**, although average lumen sizes are increased.

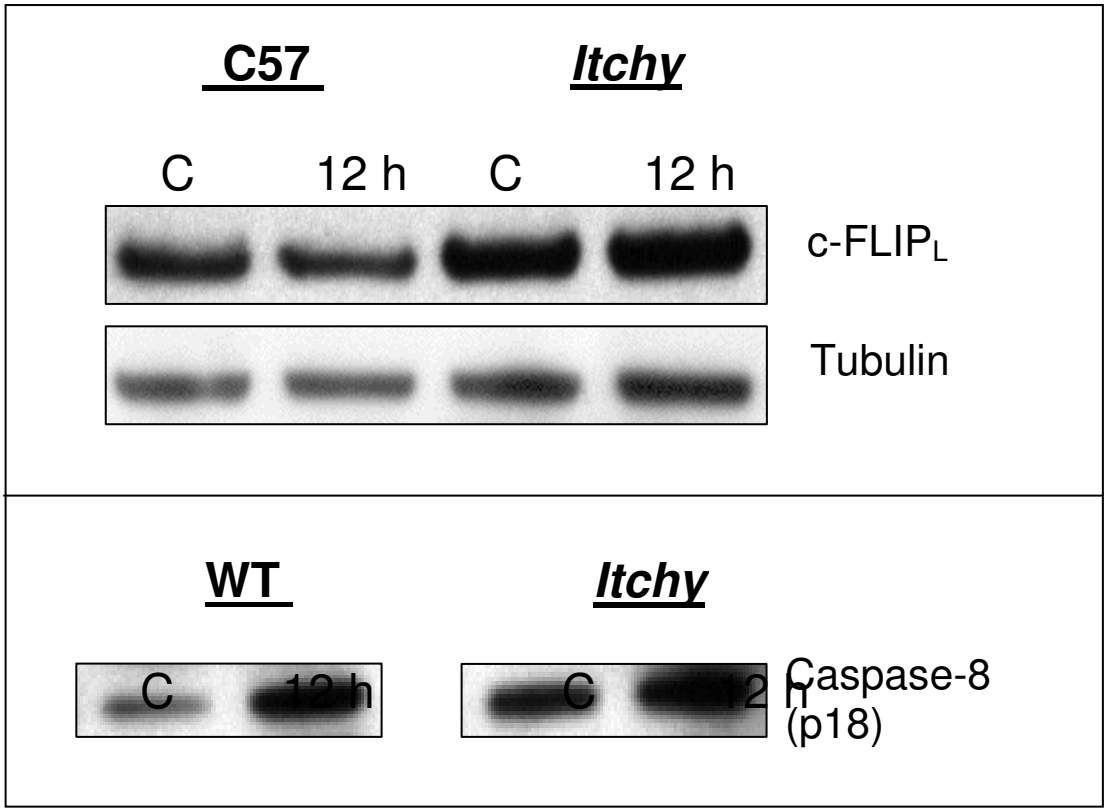

Fig. 5.5 *Itchy mice express more testicular c-FLIP$_L$ but caspase levels remain high.* The testis of *Itchy* mice express higher levels of the c-FLIP$_L$ protein as determined by western blot. Levels of active caspase-8 (p18) increase in wild type mice following MEHP. However, basal levels of p18 are high in *Itchy* control mice although MEHP-mediated morphological damage is not observed (**Fig. 5.4**).

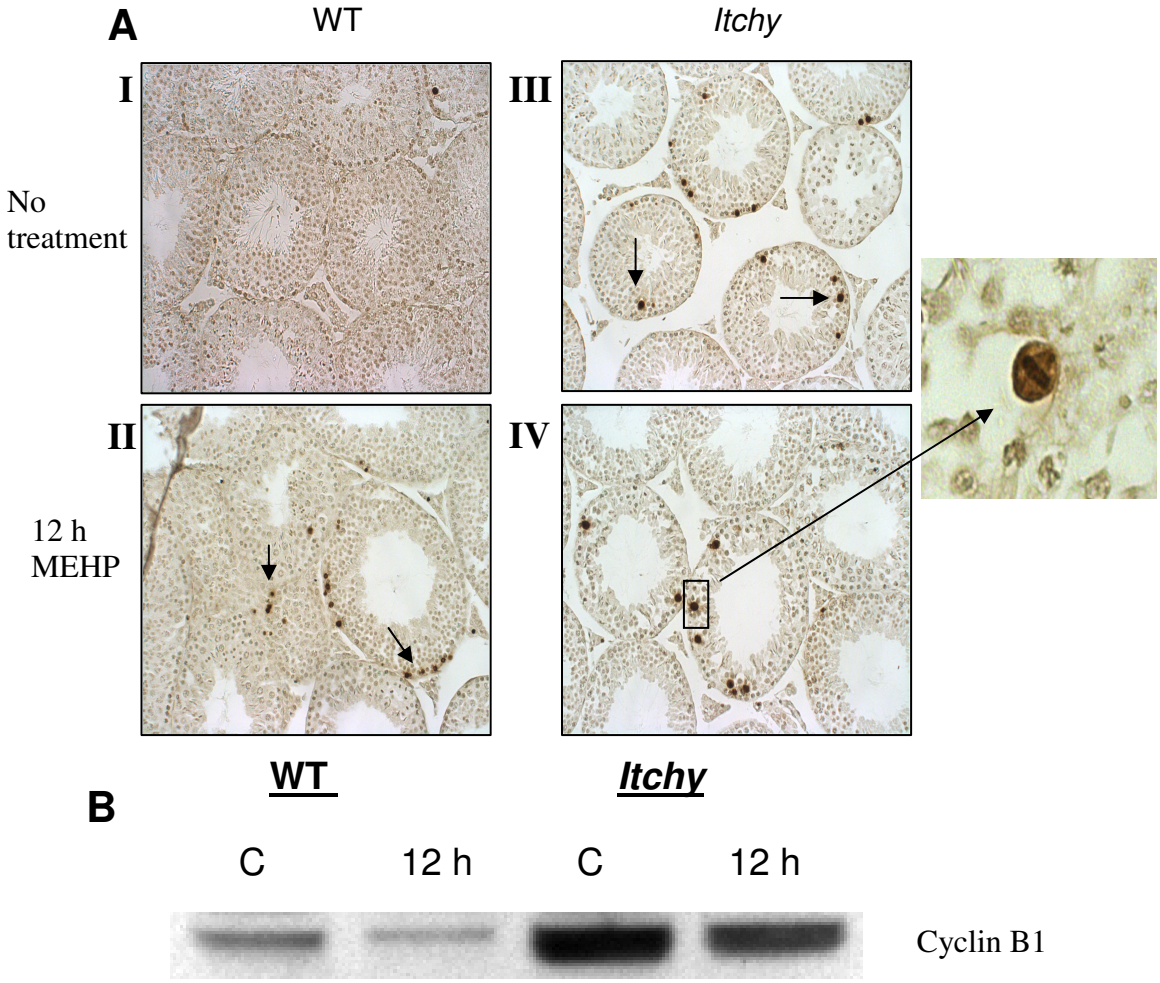

Fig. 5.6 (A) *Itchy mice show spermatocyte apoptosis distinct from MEHP mediated cell death. Itchy* deficient mice have higher rates of apoptosis, with or without MEHP treatment (III & IV). The germ cells undergoing apoptosis post-MEHP are early spermatocytes in normal WT mice (II). In *Itchy* mice, cell death is large pachytene spermatocytes and are concentrated (>50%) in meiotic cells undergoing metaphase (expansion). **(B)** *Itchy mice express elevated cyclin B1.* The cycle checkpoint protein cyclinB1 is associated with metaphase blocking. CyclinB1 protein concentrations are higher in *Itchy* mice compared to wild type, indicating an inability of the cells to progress into anaphase.

F. *Itchy* mice express more c-FLIP$_L$ but are susceptible to apoptosis through meiotic arrest

Western blots of *Itchy* testicular lysates show an increased expression of c-FLIP$_L$ protein as compared to wild type tissues, indicating that Itch may interact with and regulate the level of the protein (**Fig. 5.5**). Moreover, activated cleaved caspase-8 (p18) was detected substantially elevated after MEHP exposure in wild type mice, whereas *Itchy* mice did not exhibit a large change in caspase activation post treatment (**Fig. 5.5**). However, caspase levels in *Itchy* were already high despite increased c-FLIP$_L$ levels, suggesting the extrinsic apoptotic program was engaged without addition of the toxicant. These results could indicate that apoptosis is occurring independent of MEHP-mediated toxicity and may be the result of another physiological factor.

To determine if c-FLIP$_L$ protects *Itchy* mice from apoptosis, *in situ* detection of DNA fragmentation (TUNEL) was performed on adult Itch deficient mice and adult wild type controls, with and without a 12 hour MEHP treatment. Wild type mice showed apoptosis in early (possibly leptotene) spermatocytes of stage 7 or 8, the sub-population of germ cells most sensitive to MEHP (**Fig. 5.6**, panel II). Microscopic analysis shows that apoptosis was more prominent in slightly more mature spermatocytes, such as the pachytene spermatocyte sub-populations, in *Itchy* mice. (**Fig. 5.6**, panels, III & IV). In addition, the seminiferous tubules of the *Itchy* mice demonstrated higher basal levels of cell death in non-treated controls than wild type mice treated with MEHP for 12 h. However, the addition of MEHP did not significantly increase the number of apoptotic tubules in *Itchy* animals. The results indicate that MEHP may be inducing apoptosis through a different mechanism than the overall loss of Itch.

To investigate if the apoptotic germ cells showed different phenotypes, a closer microscopic observation and analysis of *Itchy* apoptotic cells reveal that greater than 50% are arrested at metaphase (**Fig. 5.6**, expansion). Levels of round spermatids, the germ cell formed after meiotic division occurs in spermatocytes, are also lower in *Itchy* mice. Such a result would indicate that Itch may be

essential for the completion of the meiotic process. Several proteins are required for transmission from metaphase to anaphase in meiosis, particularly cdc2 and cyclinB1. To test if the removal of Itch correlated with changes to either of these proteins, *Itchy* lysates were run on a western blot and probed with an antibody to cyclin B1. Cyclin B1 is a cell cycle checkpoint protein that must be degraded for the progression from metaphase to anaphase in testicular germ cell meiosis (Chapman, 1994). Cyclin B1 was higher in the c-$FLIP_L$ mice compared to the wild type mice (**Fig. 5.6B**). In both wild type and *Itchy* mice, addition of 12 h MEHP treatments slightly lowered cyclin levels, perhaps due to germ cell death associated with the toxicant.

G. RNAi of ITCH in GC-2 cells promotes apoptosis

To determine whether dysfunctional Itch protects from or sensitizes apoptosis *in vitro*, GC-2 spermatocyte-like cells were utilized. Disruption of Itch mRNA in GC-2 cells was accomplished using a specific siRNA delivered by an amine tranfection vector. Two principle concentrations of Itch siRNA were used, 1 μM and 100 nM. The knockdown was effective at both p53 permissive (32°C) and p53 non-permissive (37°C) temperatures (data not shown), with the 1 μM concentration especially effective. The experiments showed high levels of apoptosis associated with Itch knockdowns at both temperatures as determined by visual inspection (observation). GC-2 cells grown under at either temperature showed elevated incidence of apoptosis as determined by annexin-PI flow cytometry (**Fig. 5.7**). When p53 is minimally expressed, removal of Itch induced moderate levels of apoptosis. However, when p53 is active Itch deficient cells are sensitized to especially heavy apoptosis. Thus, the presence of active p53 may play a significant role in the apoptosis of Itch deficient mice, although the mechanism is unknown at this time. Thus, in the event that Itch is reduced or removed to the system, a redundant pathway to apoptosis may in fact be triggered, such as through cell cycle arrest.

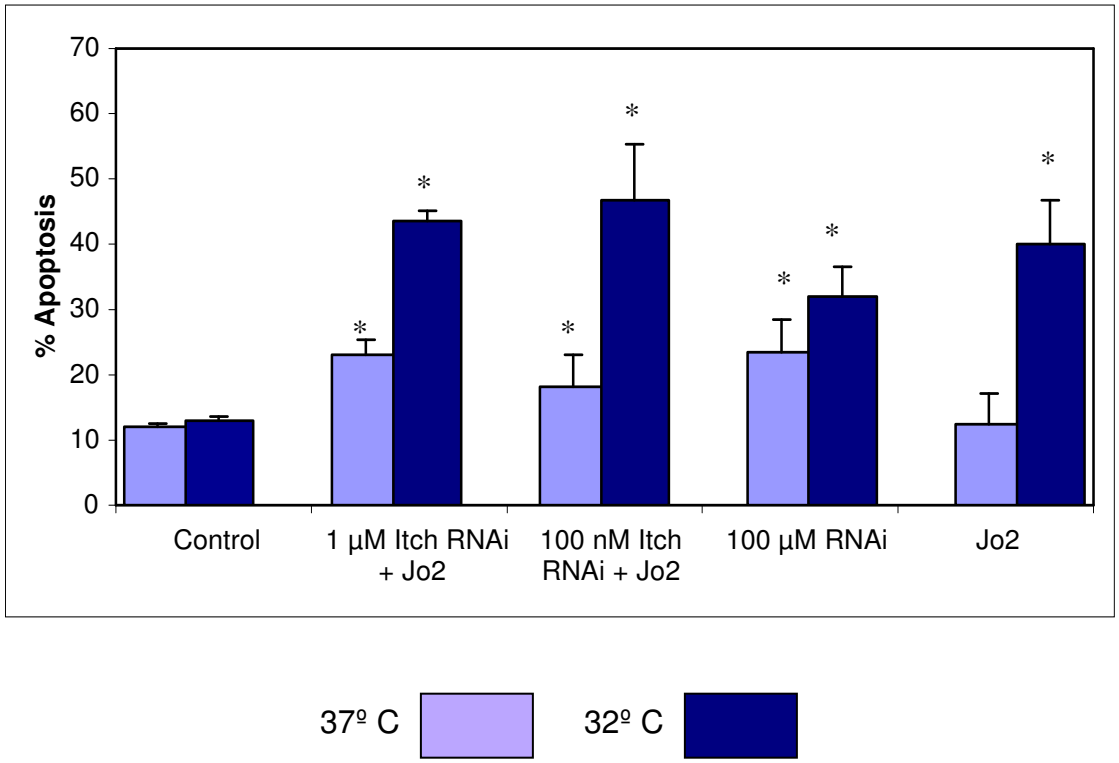

Fig5.6 *RNAi of ITCH in GC-2 cells promotes apoptosis*. Knockdown of the *Itch* gene results in higher levels of apoptosis in GC-2 cells at concentrations as low as 100 µM. Treatment with Jo2 (5 µg/ml) increased the overall apoptotic effect. Cell death is especially prevalent in cells expressing high levels of p53. Statistical significance was obtained using ANOVA and Fisher's PLDS. The graph represents three separate experiments.

5.3 Discussion

Many short-lived proteins are regulated through the ubiquitin-proteasome system of degradation. Ubiquitinylation, together with phosphorylation, represents the most common method of post-transcriptional protein regulation in the cell. c-FLIP is an anti-apoptotic protein that functions as a competitive inhibitor to caspase-8 at the DISC (Irmler, 1997). The protein is also subject to rapid turn-over in the cell and past studies have shown that over-expression of c-FLIP is associated with tumor progression (Wajant, 2003). Therefore, cellular mechanisms must be in place to prevent inappropriate expression of the protein, such as by ubiquitinylation.

In most cells c-FLIP is expressed as one of two (or both) forms, long (56 kD) and short (28kD), although a third isoform (c-FLIP-R) has been detected in leukocytes only (Golks, 2005). In mouse testis lysates, the long isoform was predominantly detected, with very weak staining of the short form (**Fig 5.1**). c-$FLIP_L$ is known to heterodimerize at the DISC with procaspase-8, leading to both a c-$FLIP_L$ cleavage (producing a p43 product) and the first procaspase cleavage (producing its own p43/41 and p12 products). The c-$FLIP_L$ p43 fragment remains bound to FADD and prevents any further procaspase-8 from binding to the DISC. The short form (c-$FLIP_s$) lacks a death domain so no cleavage occurs and the short protein only is detected (Scaffidi, 1999).

Full-length c-$FLIP_L$ (55 kD) was detected in mouse wild type testicular tissues and its level decreased in response to MEHP. An evaluation of c-FLIP cleavage products shows that the p43 processed fragment of the long form is generated with or without the addition of MEHP. The prominent banding pattern of cleaved fragments suggests that c-$FLIP_L$ resides preferentially at the DISC, where it is processed once (**Fig. 5.1**). $FLIP_s$ was not significantly detected in the testicular lysates analyzed. Therefore, $FLIP_L$ is the major isoform found in the testis and is subject to post-transcriptional modifications such as ubiquitinylation.

The level of activated caspase-8 increases in the testis of mice treated with

MEHP, with the active fragment (p18) most prominently observed at 12 hours (chapter three). The processing of c-FLIP$_L$ remained high in the control, but was decreased following MEHP. Thus, less full-length c-FLIP$_L$ may reside at the DISC following toxicant exposure, possibly due to degradation. This effect is lessened in p53-/- mice treated with MEHP, again suggesting a link between the p53 protein and apoptosis in germ cells. The reduction in processed c-FLIP$_L$ in this case could suggest that less caspase-8 is competing for space within the DISC. Alternately, less DISC may be formed without the increase in death receptors induced by p53 activity, as discussed in previous chapters.

The degradation of c-FLIP$_L$ by the proteasome was initially proposed by Fukazawa *et al* in the investigation of colon cancer cells and their resistance to apoptosis (Fukazawa, 2001). Other studies examining mouse testis detected lower c-FLIP$_L$ levels when death receptor-mediated apoptosis was induced (Chandrasakaran, 2005). To determine if FLIP$_L$ is being degraded post-transcriptionally, we performed immunoprecipitation experiments to detect ubiquitinylation of the protein in GC-2 cells after Jo2 exposure at both permissive (32° C) and non-permissive (37° C) temperatures (**Fig. 5.2**).

In cells grown at the nonpermissive p53 temperature, ubiquitin protein complexes were isolated by the anti-FLIP antibody/IgG beads and identified by a ubiquitin recognizing antibody. At 37° C, ubiquitinylation could be easily detected only after a six hour Jo2 treatment, indicating that FLIP$_L$ was less efficiently tagged for degradation in these cells (**Fig. 5.2A**). Death receptors are expressed to a lower degree at the non-permissive temperature (**Fig. 3.3B**) and seems to require additional Fas activation (by way of linking Fas trimers) to induce ubiquitinylation, accomplished by the administration of Jo2 antibody. Ubiquitinylation was much more pronounced at the permissive temperature, suggesting that the process was augmented by the activity of the p53 transcription factor (**Fig. 5.2A**). Jo2 may contribute to the overall apoptosis in the cells by helping to organize the Fas receptors into clusters of trimers, as discussed in the previous chapter with the MD5-1 antibody. However, the more important factor

in promoting ubiquitinylation appears to be the distinction between the p53 permissive and non-permissive conditions.

Reduction of c-FLIP levels at the permissive temperature strengthens the argument that robust apoptosis in spermatocytes is p53-dependent. Immunocytochemistry was performed on GC-2 cells using a c-FLIP antibody, under ordinary permissive and non-permissive growth conditions without a toxicant. At 37° C, when p53 expression is low, c-FLIP levels are can be easily visualized using fluorescent microscopy (**Fig. 5.2B,** left). However, increased p53 at 32° C appears to quench the c-FLIP signal, indicating that the protein has been reduced, presumably by ubiquitinylation (**Fig. 5.2B,** right). However, to date little data has been compiled as to what E3 ligase may act to ubiquitinylate c-FLIP.

One such candidate would be Itch, which possesses an E3 ligase of the HECT family. HECT E3 ligases can receive ubiquitin molecules from a conjugating (E2) enzyme and catalytically pass them onto a protein targeted for modification with a high level of specificity (Sun, 2003). Itch expression effects an interesting range of target proteins, including the hematopoiesis regulator Notch1 and epithelial cells (Perry, 1998). The latter specificity manifests a phenotype in Itch protein deficient animals, called *Itchy,* because the mice develop a habit of frequently scratching themselves. *Itchy* mice are moderately fertile but die after four to six months due to inflammatory lesions (Matestic, 2006). Thus, Itch activity impacts the regulation of immune response, including marking self-recognizing T-cells for degradation.

Recently Itch has been associated with apoptosis through the TNF-alpha pathway of apoptosis (Chang, 2006). Specifically, apoptosis is induced by reducing c-$FLIP_L$ levels at the DISC, possibly by increasing its ubiquitinylation though association with the Itch E3 ligase. The prospect that Itch could be involved in the regulation of c-$FLIP_L$ in spermatocytes was logical given that MEHP increases Fas, a receptor closely related in structure and function to TNF-alpha. In western blots analysis of MEHP-treated mice testis, Itch was expressed at all time points (**Fig. 5.3A**, Itch panel). c-$FLIP_L$ levels decrease modestly at

concurrent time points, supporting the hypothesis that increases in Itch correlate with its degradation (**Fig. 5.3A**, c-FLIP$_L$ panel). In addition, immunofluorescence of treated tissues showed significant increases in Itch localization to the spermatocyte cytoplasm, the potential site of c-FLIP$_L$ ubiquitinylation, and perhaps increased protein expression (**Fig. 5.3B**). Itch is known to undergo self-targeting for degradation when the protein is in excess (Mouchantaf, 2006). When active, such as under stress conditions, auto-ubiquitinylation may decrease.

To further assess Itch activity, its phospho-activation was examined. Itch phosphorylation is dependent on the MAP kinase JNK1 and is essential for the functional activity of the protein (Gallagher, 2006). At least two of three amino acid residues (ser-199, thr-222, ser-232) must be phosphorylated for the Itch E3 ligase to be functional. Analysis of MEHP treated tissues documented significant increases in Itch phospho-threonine-222 as the time course progressed to 24 hours. Whatsmore, the active phospho-JNK1 protein was detected at the same time point (**Fig. 5.3A**).

c-FLIP$_L$ degradation appears to be driven by p53 expression or stabilization. Comparisons were made between wild type and p53-/- mice after both were treated with normal doses of MEHP (**Fig. 5.3B**). Phospho-JNK1 levels were not increased in p53-/- mice, while Itch phospho-threonine-222 was minimally increased in compared to the wild type, possibly lending support that Fas expression can drive MAP kinase activity (Goillot E, 1997), (Cahill MA, 1996), (Muhlenbeck F, 1998). Furthermore, in these animals changes in both c-FLIP$_L$ and Itch were static.

Itch protein deficient mice (*Itchy*), were obtained to further assess the physiological role of Itch in germ cells. Morphological staining shows that *Itchy* mice have lower germ cell numbers (**Fig. 5.4C**). The germ cell loss is coincident with diminished populations of mature spermatocytes and spermatids, suggesting they are being reduced during a physiological process associated with the lack of Itch expression. Administration of MEHP produces some morphological derangement in the seminiferous tubules of the *Itchy* mice, such as increased

lumen sizes, in addition to the germ cell loss. However, it is important to note that they lack the extensive structural damage observed in MEHP treated tubules of wild type mice, such as widespread vacuolization, considerable Sertoli cell cytosol reduction, and missing stages. Thus, *Itchy* mice are at least partially protected from MEHP-mediated morphological damage.

Lysates from the testis of *Itchy* animals showed comparatively higher amounts of c-FLIP$_L$ than did wild type animals (**Fig. 5.5**). Tissues from *Itchy* mice were subsequently subjected to TUNEL to test the hypothesis that higher c-FLIP levels will protect germ cells from MEHP-mediated apoptosis (**Fig. 5.6**). Wild type mice underwent typical apoptosis seen previously after 12 hour MEHP exposures, with apoptosis confined usually to the furthermost edge of the basement membrane. This is characteristic of tubules with damaged Sertoli cells; spermatocytes are closer to the basement membrane because Sertoli cell cytoplasm is reduced and the lumen has increased. Furthermore, vacuolization is evident where germ cells have been lost and the stages have been disorganized.

However, TUNEL assays showed higher apoptotic rates in the control *Itchy* mice animal (**Fig. 5.6A**, panel III) than in the wild type mice treated with 12 hours of MEHP (**Fig. 5.6A**, panel II). Cell death levels did not differ significantly between *Itchy* or *Itchy* plus MEHP, supporting the previous data (**Fig. 5.5**) that mice without the functional protein were protected from the toxicant. A careful count of positive TUNEL-stained cells showed that over half (53%, observation) of the apoptotic cells were frozen in metaphase occurring in pachytene spermatocytes (**Fig. 5.6A**, insert). MEHP produced a much smaller number (10%, observation) in wild type mice and mostly in less mature leptotene spermatocytes. This novel observation implies a link between Itch and the transition between metaphase and anaphase during meiosis. Therefore, it is reasonable to see higher apoptosis in pachytene germ cells, the site of metaphase during spermatogenesis.

Itch deficient tissues expressed a significant level of cyclin B1, a protein which associates with cdc2 in the cell cycle (**Fig. 5.6B**). Cyclins help to activate cyclin dependent kinases (cdks) through phosphorylation (Gautier, 1990), (Desai,

1992). Cyclin B1 must be degraded prior to the movement of the cell from metaphase to anaphase during the cell cycle (Glotzer, 1991). Therefore, increased cyclin B1 present in Itchy tissues might be anticipated, given that many of the TUNEL labeled cells were frozen in metaphase. The disruption of the cell cycle would also resolve the increase in overall apoptosis in the tubules: germ cell loss could be explained by fewer passing through cell cycle checkpoints. In such a case, augmented c-FLIP$_L$ may not protect the cells as apoptosis is mediated by a dysfunction in the meiotic process. The above observations, together with the morphological data, suggests that Itch is may induce apoptosis through two distinct pathways: 1) reduction of c-FLIP$_L$ facilitating caspase activation after MEHP treatment or 2) meiotic arrest when the protein is totally removed. Thus, Itch is probably required for normal spermatogenesis

Removal of Itch *in vitro* also promotes cell death. In GC-2 cultures treated with either 1 μM or 100 nM of Itch siRNA, the Itch protein is knocked-down at both 37° C (p53 non-permissive) and 32° C (p53 permissive) temperatures as determined by western blot (data not shown). Annexin-PI flow cytometry was used to determine the apoptotic response of the GC-2 cells to Itch deficiency (**Fig. 5.7**). Cell death quantitation shows increased apoptosis in all samples treated with Itch siRNA only or in combination with Jo2. Therefore, Itch is probably required for some other, critical function within the germ cell. Keeping with previous observations, the apoptosis seen is also greater at 32°C, when p53 is properly folded and functional. Therefore, p53 plays a role in sensitizing cells to apoptosis when Itch is reduced.

The GC-2 line may be of particular use in this investigation because cells grown at the permissive temperature have been described to undergo meiosis (Hofmann, 1994) (Hofmann, 1995). The heightened apoptosis occurring at the permissive temperature after the RNAi of Itch is consistent with a disruption in meiosis and support the *in vivo* data. However, there is controversy as to whether GC-2 cells are a suitable modeling this process, as some cellular markers are not readily expressed in GC-2 cells (Wolkowicz, 1996). Until further studies are

conducted, the meiotic component of Itch in GC-2 cells is speculative. However, the data suggests that p53 activity may play a role in promoting apoptosis following knock-down in GC-2 cells. One possibly is that p53-transcribed proteins such as Fas of Puma aid in clearing cells targeted to undergo apoptosis *via* disruption of the cell cycle.

Evidence compiled both *in vivo* and *in vitro* support the concept that Itch activation can be induced in germ cells following MEHP exposure and that p53 levels contribute to this process. Itch activation is correlated with c-FLIP$_L$ reductions, whether directly or through an intermediate protein. In addition, experiments in *Itchy* mice support this finding in that they are partially protected from MEHP-mediated germ cell damage. However, complete removal of Itch from the testis promotes germ cell apoptosis, possibly through the cell cycle, suggesting a novel, as yet undescribed function for the protein.

Chapter 6:
Concluding Remarks

Functional spermatogenesis is the cornerstone of male reproductive health. Misregulation of this process is detrimental to the ability of the testis to produce germ cells and mature sperm, leading to syndromes such as testicular dysgenesis and, in extreme cases, infertility. Germ cell numbers are limited by physiological conditions and the support of Sertoli cells within the seminiferous tubules. In order to maintain an optimal population, a certain number of these cells are routinely eliminated by apoptosis during spermatogenesis. However, cell death is inappropriately increased by toxicant-induced stress and lead to a variety of testicular injuries. Understanding the mechanisms of stress signaling and apoptosis in relation to these conditions is therefore of significant physiological importance.

DEHP is an environmental toxicant, whose major metabolite, MEHP, causes testicular injury. MEHP is unusual because it uniquely targets and damages the Sertoli cell, a source of nutrients and growth factors for germ cells, rather than the germ cell itself. When the paracrine signaling relationship between both cells are disrupted, a disturbance in spermatogenesis can occur. Sertoli cell injury may impair the spermatogenic process by either removing the structural support required for germ cell motility and orientation or by withdrawing factors needed for maturation. Death ligands such as FasL or TRAIL are also expressed by injured Sertoli cells. In each case, the germ cell is able to detect changes in Sertoli support, prompting the engagement of the apoptotic process.

One of the aims of this dissertation was to evaluate possible mechanisms of how germ cells detect stress in response to Sertoli cell injury and through what cellular determinants subsequent apoptosis is achieved. The studies revealed several novel aspects of germ cell stress signaling, including the observation that p53 is a requirement for robust apoptosis in response to MEHP. The

transcriptional activity of the p53 protein was shown to be correlative with death receptor expression and caspase activation both *in vivo* and *in vitro*.

The increased activity of p53 is one area of inquiry that could be investigated further. Preliminary experiments demonstrated the novel result that MEHP can elevate p53 protein levels *in vivo*. The p53 protein is normally kept at a low level by its direct inhibitor, MDM-2 (Momand, 1992). In order to increase protein concentrations in the cell, p53 must be stabilized. Commonly, its degradation can be forestalled by post-transcriptional phosphorylation on one of more of its amino acid residues. These modifications stabilizes p53, either by reducing its association with MDM-2 (such as phosphoserine-15 or -20, -18 in mouse) or preventing its translocation from the nucleus (phosphoserine-392). (Kim, 2004), (Ashcroft, 1999) Our data reflects the latter as a strong possibility, given that the -392 residue is phosphorylated in the wild type control and increases upon MEHP treatment.

The significance of more p53 remaining in the nucleus can be witnessed by the increases of Fas expressed post-MEHP exposure *in vivo*. The genes for death receptors Fas and DR5 are known to possess p53 response elements. In fact, death receptors were shown to be increased upon MEHP treatment *in vivo* or simply by inducing p53 activity in GC-2 cells. The data supports previous studies in which MEHP induces the extrinsic pathway upon administration (Lee, 1997), (Boekelheide, 1998). A more complete evaluation of p53 post-transcriptional modifications may shed additional light on how MEHP influences the transcription factor. Certain amino acid residues, such as threonine-81 and serine-6, stabilize p53 and are critical for inducing apoptosis (Oleinik, 2007), (Buschmann, 2001)

The role of p53 in testis likely extends beyond stress signaling and into the regulation and development of spermatogenesis as well. The expression of p53 is important in the activity of UCH-1/3 (ubiquitin c-terminal hydrolase 1 and 3), a pair of enzymes particularly active in the physiological “first wave” of apoptosis experienced in the few weeks of spermatogenesis (Kwon, 2005). UCHL enzymes

help to maintain a cytosolic pool of ubiquitin. Mice lacking UCHL's have express less active p53 and a reduced ability to initiate apoptosis by targeting cells for degradation. The resulting accumulation of damaged or non-functional germ cells effectively inhibits levels of normal spermatogenesis (Kwon, 2004). Thus, under ordinary conditions p53 could act to rescue testicular tissues from cells that interfere with normal sperm production. Other p53-dependent proteins may fill similar functions. For example, another gene under the transcriptional control of p53, *puma*, has also been shown to activate in the physiological pruning of early gametes in the first wave (Lizama, 2007). An emerging role for p53 family proteins p63 and p73 can has also been seen in spermatogenesis and testicular development. In particular, p63 is becoming established another factor in shaping germ cell populations in young animals and may be associated with apoptosis under some conditions (Petre-Lazar, 2007), (Sayan, 2007). Therefore, an investigation into this initial stage of spermatogenesis may elucidate the responsibility of p53 to aid in germ cell proliferation in conjunction with its apoptotic abilities.

The preliminary experiments with p53 uncovered an important additional finding, reported for the first time, that the TRAIL/DR5 system is able to engage apoptosis in germ cells. Additionally, the use of a DR5 agonistic antibody (MD5-1) effectively increased the overall cell death. The role of these proteins in combating tumor cells is of significant interest and has been the subject of many chemotherapeutic studies. The reasons explaining TRAIL/DR5-mediated apoptosis is often specific for tumorigenic cells is unclear, particularly given its wide tissue distribution and expression. The experiments in GC-2 cells designed to model the action of MEHP, in which death ligands (TRAIL) are expressed by the Sertoli cell after injury and interact with neighboring germ cells. The data suggests that DR5 is activated by p53 transcriptional activity and shuttled to the membrane, where anti-DR5 antibodies link groups of receptors, possibly visualized by confocal microscopy in chapter four. These groups have been previously seen in Jo2-augmented Fas "capping" and are often components of

lipid rafts (Cremesti, 2001), (Grassme, 2001). Whether or not DR5 is truly experiencing capping is not certain, though the data supports that p53 status and death receptor expression is up-regulated in response to stress signaling.

Perhaps more importantly is the recognition that stress can institute a TRAIL/DR5 program of cell death in normal physiological cells. Extensive research has been dedicated to the coupling of TRAIL with agonistic antibodies in an effort to halt tumor progression (Uno, 2006), (Takeda, 2004), (Ichikawa, 2001). A little explored side effect is the inappropriate prompting of apoptosis in certain highly proliferative cell populations that are not cancerous. Thus, in tissues such as the testis, engagement of the TRAIL/DR5 system may induce cell death in normal cells in addition to those that are cancerous. A supplementary study in other proliferative tissues expressing TRAIL may yield similar results and could potentially restrict the safe use of chemotherapeutic mixtures.

The impediment of apoptosis through the extrinsic pathway is often through the caspase-8 inhibitor c-FLIP. The ability of the protein to saturate procaspase-8 binding sites at the DISC and inhibit its activation is well established and seen in many diverse cell types. However, its regulation has been in large part limited to the knowledge that it is a fast turn-over protein likely controlled through ubiquitinylation. We report here that c-$FLIP_L$ is reduced in response to MEHP in mouse spermatocytes and is ubiquitinylated in the spermatocyte-like germ cell line GC-2. Furthermore, the degradation is linked to the activity of p53. From chapters three and four, it was shown that stress applied to either mice or cultured cells will induce p53-dependent death receptor expression, which in turn shifts the homeostatic balance of germ cells to apoptosis.

We sought to further understand how c-$FLIP_L$ could be degraded by determining if certain ubiquitin E3 ligase would initiate this process. The E3 ligase specific for its degradation is not known. Itch was chosen as a candidate due to its involvement in the TNF-alpha -mediated ubiquitinylation of c-$FLIP_L$ in liver cells, the first study to directly implicate a specific E3 ligase in its

degradation. TNF-alpha is a ligand that reacts with its receptor, TNFR, and is similar in action to the Fas /FasL system promoted following MEHP treatments. Hepatocytes are also a cell type that undergoes TRAIL-mediated apoptosis under physiological conditions. Only one study had investigated Itch in the testis and the focus of their investigation was in the Sertoli cell tight junctions and not in germ cells (Lui, 2005).

Itch activity is increased in response MEHP and appears to play a vital role in testicular apoptosis (chapter five). However, its direct role in ubiquitinylating c-FLIP remains to be validated, and other E3 ligases may regulate the protein. One possibility that has been advanced is TRAF2 (TNF-R associated factor 2), which associates and decreases c-$FLIP_L$ in response to TRAIL-mediated apoptosis in tumor cells (Zhang, 2004) TRAF2 also migrates to lipid rafts, where Fas and DR5 signals are often elevated amplified, and may aid in c-FLIP ubquitinylation after T-cell activation (Misra, 2007). However, the traditional extrinsic pathway may not be employed, as there some evidence that death receptors are expendable in this reaction. TRAF2 and its association with threonine/serine kinase RIP and has been implicated in the activation of caspase-8 and cell cycle arrest (Jin, 2006). A new E3 ligase, MEX (MEKK1-associating), may show promise as a regulator of c-FLIP due to its co-expression with Fas and DR5, and activation of caspase-8 (Nishito, 2006). In addition, MEX is predominately expressed in the testis.

Itchy mice showed higher levels of c-$FLIP_L$ and partial protection from MEHP. However, an intriguing observation in these animals is that removal of Itch expression actually promotes death in germ cells as well, independently of MEHP (unpublished observations). Caspase-8 activation is high in untreated *Itchy*, which correlates with high basal apoptosis levels. Furthermore, the microscopic examination reveals that most of the apoptotic cells are frozen in metaphase and immunoblotting of shows that cyclin B1 degradation is impaired when Itch is not functional. Thus, Itch may play a previously undescribed role in the cell cycle progression. A knockdown of Itch in GC-2 cells also promoted cell

death. Interestingly, p53 expression was important in apoptotic levels. Since p53 is a regulator of the cell cycle in addition to apoptotic signaling, its influence would justify a potential investigation into this area.

Taken together, the observations open a new and thus far unstudied aspect of Itch in the testis. Spermatocytes are the site of meiosis in spermatogenesis and also the germ cell subtype most reliant on the proper function of the Sertoli cell. That pachytene spermatocytes are targeted for apoptosis is logical, since they are the site of recombination and take the longest to transition during the cell cycle. Therefore, these cells are likely the most sensitive not only to changes in Sertoli cell signaling/apoptosis but also alterations in proteins that regulate the process of meiosis itself. There is little information on testis specific E3 ligases and their interaction with germ cell meiotic proteins.

The influence of MEHP on the meiotic process would be a fertile field of investigation and further uncover details on why germ cell numbers are reduced under stress conditions. The anaphase promoting complex (APC) has been established as the source of ubiquitinylation and degradation of cyclin B1 (Glotzer, 1991). Interestingly, the APC also uses an E3 ligase to induce degradation. The data compiled here suggests that the Itch E3 ligase may be used for ubiquitinylation of substrates during this stage of the cell cycle as well. Other ligases have been discovered in meiosis and spermatogenesis, such as shia1, which may control aspects of chromosome segregation, and promote a metaphase block (Dickins, 2002). Like Itch, Shia1 deficient mice are fertile but are but display difficulty transitioning through the meiotic phases, possibly resulting in meiotic arrest.

Perhaps Itch acts to regulate one or more of the proteins involved with the orderly progression of the cell cycle during spermatocyte progression. There is enormous redundancy in cells; Itch may act to aid APF under cellular stress or another physiological condition. This function could prove to be an interesting continuation of the work done in spermatocytes, which are often the most

sensitive germ cell to apoptosis, not only after MEHP, but a wide range of toxicants.

The studies to this point have suggested p53 as critical first response to germ cell stress. Our data has firmly established a link between the ability of MEHP to promote apoptosis and activate p53. The hypothesis that p53 is a sensitizer of germ cell stress has been supported in GC-2 cells and with p53 deficient mice, which express fewer death receptors, reduced caspase-8 activity, and lower incidence of apoptosis. The transcriptional targets of p53, death receptors, likely drive apoptosis and effect c-$FLIP_L$ degradation. The literature shows that death receptors can promote JNK1 activity by stimulating upstream kinases: MKK4 and MKK7 can be induced by Fas and TNF-alpha respectively (Toyoshima, 1997), (Moriguchi, 1997). Hence, our experiments suggest that upstream signaling factors that activate JNK1 activation may be triggered by MEHP treatments.

However, an important note is that p53 can be stabilized by MAP kinases. MAP kinases are serine/threonine kinases that have been linked to many different cellular processes including the regulation of cell cycle progression, proliferation, differentiation, and cell death (Bogoyevitch, 2006). The mechanism bears examination, particularly since p53 is a phosphorylation target JNK1, which also activates Itch. A further line of research would be to identify if a particular MAP kinases influences the stability of p53 in addition to activating Itch. For example, future experiments could use JNK knock-out mice to determine if any protection is observed in the testis. In addition, knowing which upstream activators of JNK1 could produce clues as to the external stimuli that are ultimately responsible for initiating the MAPK kinase cascade in germ cells following MEHP-induced Sertoli cell injury.

Our studies have made important advancements in identifying proteins intimately involved with the MEHP-mediated toxicity of germ cells. Our initial hypothesis that p53 participates as a monitor of cell stress apoptosis has been supported experimentally both in rodent *in vivo* models and in culture. Of

particular note is that spermatocytes, often considered to be the most sensitive population to stress induced apoptosis, are also readily killed by the TRAIL/DR5 system and that p53 in part mediates this reaction. Our work has also uncovered the potential importance of Itch in germ cell homeostasis. While the original idea that Itch mediates c-FLIP$_L$ degradation received some support, additional experiments will have to be completed to convincingly establish this. However, the possible role of Itch in meiotic progression is a novel observation that may explain why spermatocytes specifically undergo apoptosis in knockout mice. The conclusions of the research have been summarized in the germ cell stress model (**Fig. 6.1A/B**).

The ultimate purpose of this research is to explain the mechanism of MEHP in male infertility. The supportive capacity of the Sertoli cell is known to be reduced when damaged by MEHP. MEHP is thought to induce derangements in the seminiferous tubules collectively known as testicular dysgenesis syndrome, a possible precursor to male infertility. Our study had addressed the downstream effects of this injury and its impact on the most prominent cell in the adluminal compartment, the spermatocyte. The homeostasis of these cells plays a key part in the on-going ability of the testis to produce mature sperm and their mechanism of apoptosis is integral to understanding how spermatogenesis is regulated in response to toxicant stress.

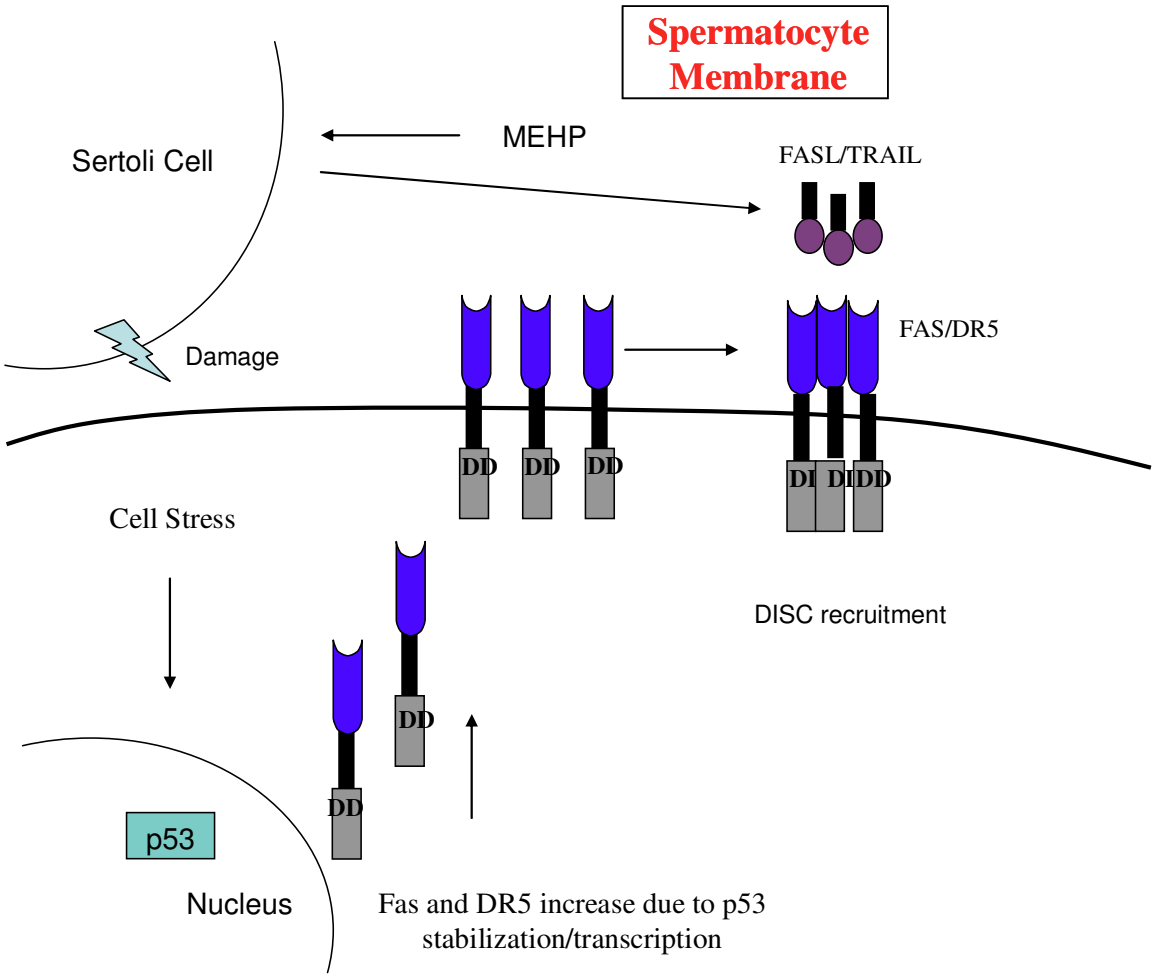

Fig. 6.1A *Summary Figure*. Testicular treatments of MEHP cause Sertoli cell damage and ensuing apoptosis in the germ cell. The p53 protein is stabilized in response to cellular stress and translocates to the nucleus where death receptor transcription is increased. Death receptors are trafficked the membrane, where they associate and interact with death ligands released by the Sertoli cell.

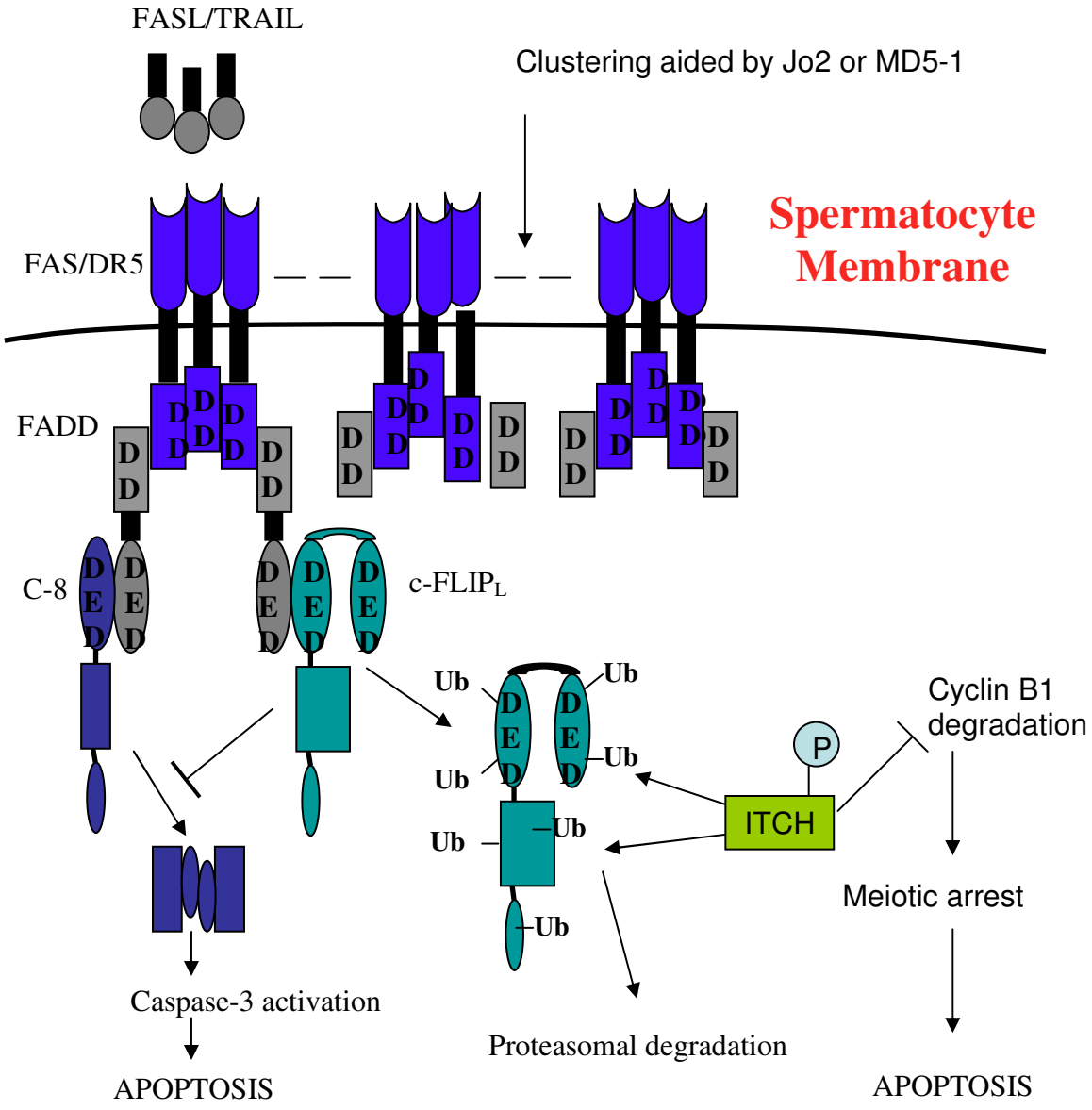

Fig. 6.1B *Summary Figure*. Triggering of death receptor/ligand complexes promote DISC formation and apoptosis through the extrinsic pathway. Apoptosis can be blocked by c-FLIP$_L$. The E3 ligase Itch can potentially promote apoptosis through ubiquitinylating FLIP$_L$. However, removal of Itch also induces cell death, possibly by meiotic arrest.

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Vita

Chad Marcus McKee was born in Jacksonville, Florida on June 4th, 1975, the son of Susan Lynn McKee and James Marcus McKee. After graduating from Walton High School in 1994, he attended Kennesaw State University where he graduated with a Bachelor of Science in Biology in 2000. Over the next two years, he worked at the CDC (Centers for Disease Control) on a pre-doctoral research scholarship from Oak Ridge National Laboratories (DOE). Chad then enrolled at the University of Texas-Austin, graduate program of Cell and Molecular Biology, and joined the laboratory group of Dr. John Richburg. Chad was awarded the Toxicology Training grant (2004-2007) and earned First Place in Platform Competition of the Gulf Coast Society of Toxicology meeting (2007). He has also presented his work at the Society of Toxicology (2006) and Federation of American Societies for Experimental Biologists (FASEB) (2007) national meetings. His listed as co-author of a Biology of Reproduction paper from 2005 and as primary author on an Apoptosis paper from 2006.

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